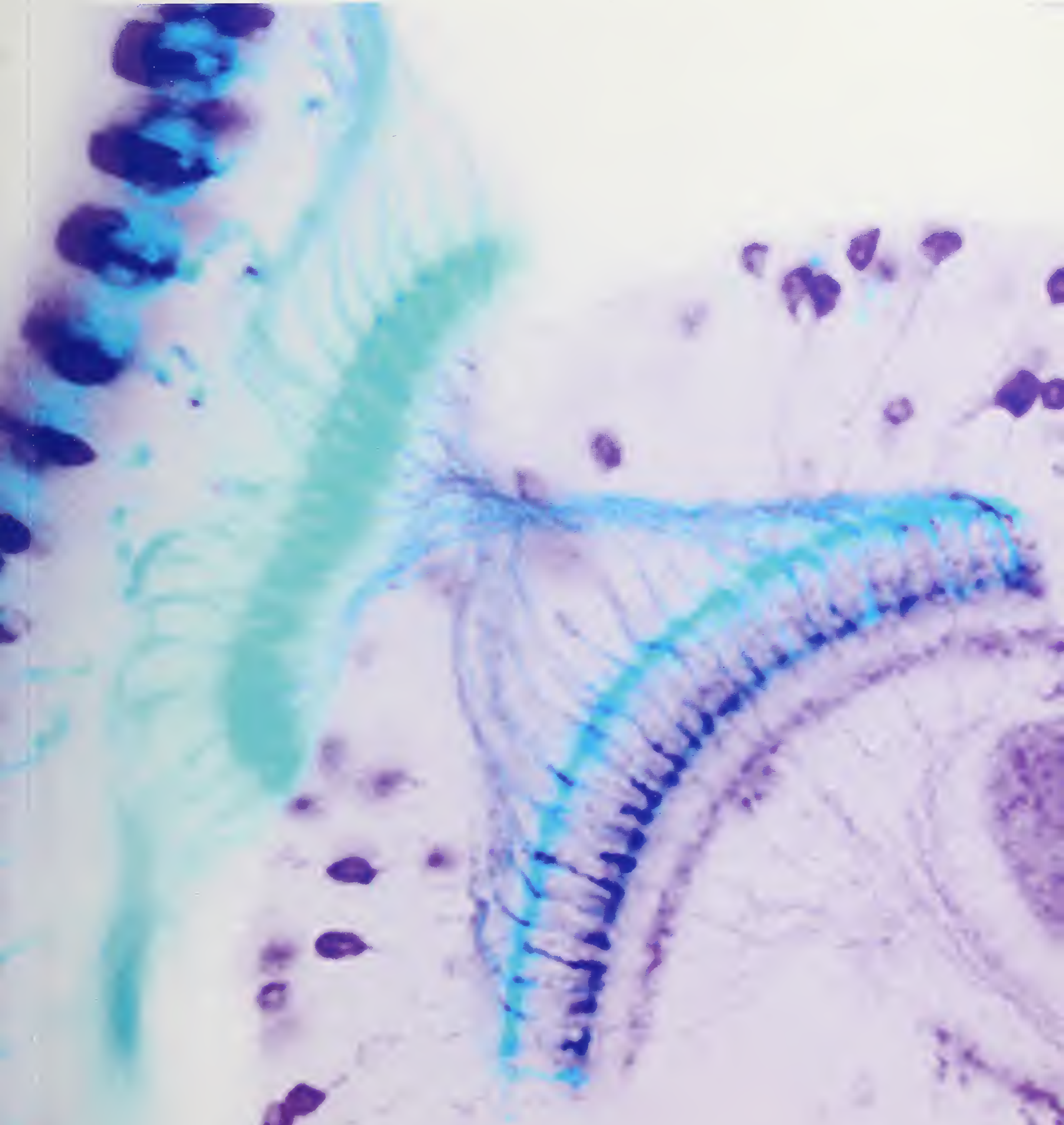
# 2002 Annual Report of the Division of Intramural Research



National Institute of Child Health and Human Development

## COVER

Confocal imaging of a developing Drosophila optic lobe, illustrating the basic wiring scheme of the visual system. Photoreceptor neurons in the retina (left upper corner) extend their axons into the optic ganglions to form an array-like structure. The R8 and R7 photoreceptor axons (colored in light and dark blue, respectively) terminate at two distinct layers in the medullar ganglion (center). Axons originating from a class of medullary interneurons (colored purple) project through the medulla where they form en passant synapses with R7 termini. They relay R7-mediated visual information to higher optic ganglions (right lower corner). Experiments performed by Chi-Hon Lee in the Laboratory of Gene Regulation and Development (p. 193) explore the mechanisms regulating the formation of these connections.

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# NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

## DIVISION OF INTRAMURAL RESEARCH

# ANNUAL REPORT

## 2002

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service

National Institutes of Health
Bethesda, Maryland 20892

May 7, 2003

Philip S. Chen, Jr., Ph.D.
Senior Advisor to the Deputy
Director for Intramural Research
Office of the Director
National Institutes of Health
1 Center Drive
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Bethesda, MD 20892-0151

Dear Dr. Chen:

Thought you might be interested in reading about our research programs.

Sincerely,

Owen

Owen M. Rennert, M.D.
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Division of Intramural Research
National Institute of Child Health and
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# SCIENTIFIC DIRECTOR'S PREFACE

"***The invariable mark of wisdom is to see the miraculous in the common.***" Ralph Waldo Emerson

"***Imagination is more important than knowledge, for knowledge is limited while imagination embraces the entire world.***" Albert Einstein

The guiding ideal underpinning the research programs of the National Institute of Child Health and Human Development (NICHD) holds that every individual is born healthy, is born wanted, and has the potential for a productive life unhampered by disease and disability. Human development is continuous throughout life, and optimal outcomes of development are a reflection, in large part, of episodes/events in early life, sometimes before birth. Optimal outcomes of development are important to the individual and to society.

NICHD recently developed a strategic plan outlining four areas of emphasis to achieve this potential. The areas of emphasis are:

- Developmental Biology—understanding the basic biology of normal and abnormal development from early development *in utero* through organogenesis
- Biobehavioral Development—research to understand the biology and developmental processes of cognition and behavior and the environmental events that modify them
- Genetic and Fetal Antecedents of Disease Susceptibility—the interaction of genetic, epigenetic, and cellular events, and environmental factors in the fetal and postnatal environment that contribute to health or the pathophysiology of disease
- Reproductive Biology—the biological and behavioral factors that allow couples to have healthy children

The Intramural Research Program focuses on the biological, medical, and behavioral aspects of normal and abnormal human development. Its investigative efforts span the spectrum from the study of the "physics" of prokaryotic and eukaryotic cells to research on genetic regulation in model systems (fish to non-human primates), including the development and acquisition of behavioral traits from monkey to man, to studies of the natural history of human diseases and the development of new diagnostic and therapeutic approaches. In addition, the intramural research program recognizes its obligations to train biological and physician scientists who will ensure that the fundamental discoveries in developmental, reproductive, and cellular biology, along with advances in genomics and proteomics, are applied to enhance our understanding of the processes central to "health" and to the development of new therapies against disease.

"***...no distinction can be drawn between basic and applied science; there is only science that has or has not yet been applied.***" G. Weissmann

# RESEARCH FOCUS OF THE LABORATORIES AND BRANCHES

## CELL BIOLOGY AND METABOLISM BRANCH

**Juan S. Bonifacino, Ph.D., Chief**

The Cell Biology and Metabolism Branch (CBMB) conducts studies in various areas of molecular cell biology, including the mechanisms of intracellular protein trafficking and organelle biogenesis (*Juan Bonifacino, Catherine Jackson, Ramanujan Hegde, and Jennifer Lippincott-Schwartz*), the regulation of the cell cycle during oogenesis (*Mary Lilly*), the biology of metal metabolism (*Tracey Rouault*), and adaptive responses to environmental stresses (*Gisela Storz*). The CBMB has outstanding microscopy facilities with particular capabilities in light and fluorescence microscopy. Three powerful confocal microscope systems enhance its ability to study cell structure and dynamics by using techniques such as fluorescence imaging in real time, photobleaching, fluorescence resonance energy transfer, fluorescence correlation spectroscopy, and image analysis. In addition, the CBMB has facilities for cell microinjection and micromanipulation, for automated DNA sequencing, and for work with bacteria, yeast, *Drosophila melanogaster*, and mammalian cells. Members of the CBMB apply knowledge gained from the study of basic cell-biological problems to the elucidation of the causes of human diseases, including disorders of lysosome-related organelles and iron overload.

Over the past year, scientists at CBMB have developed novel fluorescence imaging tools that promise to revolutionize the analysis of protein dynamics within cells. One of these tools is a variant of the green fluorescent protein (GFP) that shows greatly increased fluorescence after activation by light and functions under physiological conditions. Use of this photoactivatable GFP permits the tagging of proteins in a small area of the cell for selective marking by light activation so that a protein's movement through the cell can be followed against a dark background by fluorescence microscopy. CBMB also developed a novel system, based on fluorescence resonance energy transfer microscopy, to detect changes in the structure and organization of multi-protein complexes of integral membrane proteins *in situ*. This advance is now being used to visualize directly the spatial organization of protein transport into and out of the endoplasmic reticulum *in vivo*.

In other studies, CBMB scientists have discovered critical components and interactions of the molecular machinery involved in protein secretion and sorting to different intracellular compartments. The research includes the elucidation of signal-adaptor protein interactions involved in protein sorting to lysosomes and the identification of interaction partners for guanine nucleotide exchange factors for the Arf family of small GTPases. A genomic screen resulted in the identification of many new genes involved in the biogenesis of the yeast vacuole and mammalian lysosomes. The scientists also discovered a new protein complex that is defective in mutant mouse models of the pigmentation and bleeding disorder Hermansly-Pudlak syndrome.

Another CBMB group found that ablation of genes encoding iron-regulatory proteins in mice cause accumulation of iron in certain regions of the brain, with progressive development of a neurodegenerative disorder. This finding suggests a possible connection between iron overload and other neurodegenerative disorders of unknown etiology. Other studies identified important cellular regulators of oocyte development in the ovary and of the response to oxidative stress mediated by the small RNA OxyS.

## DEVELOPMENTAL ENDOCRINOLOGY BRANCH

**Carolyn Bondy, M.D., Chief**

The Developmental Endocrinology Branch investigates human growth and development. Groups led by *Carolyn Bondy* integrate basic and clinical research to elucidate genetic and physiological mechanisms governing male and female development. They have recently shown that estrogen augments brain insulin-like growth factor 1 (IGF1) expression, that IGF1 promotes brain glucose transporter expression and glucose utilization, and that enhanced IGF1 expression and associated metabolic activity augment dendritic growth and synaptogenesis, thus explaining the mental retardation found in IGF1-deleted individuals. They also conduct genotype-phenotype clinical studies on Turner's syndrome with the goal of identifying X-chromosome genes involved in sexually dimorphic development of the reproductive systems and brain.

*Greti Aguilera's* group investigates the neuroendocrine mechanisms underlying the stress response, with emphasis on the regulation of the hypothalamic pituitary adrenal (HPA) axis. The researchers have shown that exposure to a repeated somatosensory stress causes hyper-responsiveness of the HPA axis to a novel stress.

The research group of *Constantine Stratakis* focuses on genetic and molecular disorders of the adrenal cortex, with emphasis on those that are hereditary and associated with multiple tumors in other endocrine glands. The team has identified the *PRKAR1A* gene, a novel tumor suppressor gene, that, when mutated, leads to Carney complex (CNC) and related syndromes. PRKAR1A is the main regulatory subunit of protein kinase A, a central signalling pathway for many of the cellular functions and hormonal responses. The group also investigates genetic defects in related syndromes such as Peutz-Jeghers. A genome-wide screen recently led to the identification of the locus of the gene responsible for inherited adrenocortical aldosteronomas (familial hyperaldosteronism type II or FH-II).

*Jeff Baron's* group investigates the cellular and molecular mechanisms governing bone growth and development and applies its findings to improving our understanding of human skeletal disorders. The researchers found evidence that bone mineral acquisition early in life has little or no effect on adult bone mass because many areas of the juvenile skeleton are replaced *in toto* through skeletal growth, challenging the widely held assumption that decreased bone mineral acquisition during childhood causes a permanent decrease in bone mineral density that increases the risk of fractures in late adulthood.

*Lawrence Nelson's* group focuses on premature ovarian failure, which affects 1 percent of women under age 40. The team has found evidence for steroid cell autoimmunity in approximately 5 percent of these women and uncovered asymptomatic adrenal insufficiency, a potentially fatal disorder, in 3 percent of them. The scientists demonstrated that measuring adrenal antibodies is the best method for detecting asymptomatic adrenal insufficiency in affected women. They also identified the human homolog of the mouse *Mater* gene, disruption of which leads to infertility in the mouse.

*Jack Yanovski's* group, known nationally for its work on pediatric obesity, has shown that overweight and obese children are at greatly increased risk for abnormal glucose tolerance; that treatment with Orlistat is safe, well-tolerated, and moderately effective in producing weight loss in severely overweight adolescents; and that the skeletal maturation and earlier sexual development of African American children is largely accounted for by their increased adiposity.

## ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

**Kevin Catt, M.D., Ph.D., Chief**

Investigators in the Endocrinology and Reproductive Research Branch address the molecular and cellular mechanisms of reproductive hormone action, the structure-function properties of hormone receptors, and the mechanisms and roles of protein phosphorylation and sulfonation in metabolic regulation and signal transduction. The branch found that the manner in which the endogenous GnRH receptor mediates regulation of pulsatile neuropeptide secretion involves autocrine switching of receptor coupling between stimulatory and inhibitory G proteins. The LH receptor gene promoter is under tonic repression by deacetylation and is activated by depression through specific signal inputs. The HDAC-mSin3A complex regulates human LHR gene transcription by interacting with

Sp1/Sp3 and by region-specific changes in histone acetylation and Pol II recruitment to the promoter. The complex genomic structure of the human Prl receptor gene has been resolved, and the manner in which its transcription is controlled by multiple promoters with individual regulatory mechanisms is undergoing clarification. Identification of the enzyme that sulfonates cholesterol revealed two isoforms derived by differential splicing that preferentially produce cholesterol sulfate or pregnenolone sulfate.

Investigations of calcium signalling in pituitary cells explored the role of endothelin receptors in controlling voltage-gated calcium influx, the extracellular messenger functions of calcium, and the interactions between intracellular calcium and protein kinase A in the control of soluble guanylyl cyclase activity. Studies on calcium-conducting purinergic receptors have identified structural domains that determine ligand selectivity, desensitization, and recovery from desensitization. Heteromeric receptors formed from distinct subunits were found to exhibit new functions that are endowed by the participating extracellular and C-terminal subdomains.

The branch has advanced the analysis of molecular mechanisms of cell signalling through the use of fluorophor-labeled proteins. These probes permit the visualization of highly localized changes in inositol lipid levels in cellular responses ranging from ovum fertilization to phagocytosis of bacteria, and has enabled the group to visualize the active form of the oncogenic protein, Ras, and to identify the vesicular compartments through which the agonist-activated angiotensin $AT_1$ receptor is internalized and recycled to the plasma membrane.

The branch investigates the physical and functional interactions between G protein–coupled receptors (GPCRs) and growth factor receptors in neuronal, pituitary, and hepatic cells. Analysis of the complex pathways that mediate the activation of MAP kinase phosphorylation cascades has identified temporal signalling modalities that are determined by transactivation of the EGF receptor by GPCRs such as the angiotensin and GnRH receptors. These studies are directed at understanding the molecular mechanisms that determine the intensity and spatio-temporal regulation of ERK signalling and its actions on nuclear gene expression during mitogenic signalling by GPCRs.

The importance of neurogranin, a calmodulin (CaM) binding protein, in synaptic plasticity has been shown in knock-out mice with impaired learning as well as deficits in long-term potentiation and CaM kinase autophosphorylation. Both phosphorylation and oxidation of neurogranin regulate neuronal signalling during NMDA receptor activation. Thionylation of neuronal proteins provides protection against oxidative insults and acts in cell signalling. Glutathione disufide S-oxide has been identified as a highly reactive glutathionylating agent and can serve to integrate oxidative and nitrosative cellular responses through its reaction with thiol groups.

## HERITABLE DISORDERS BRANCH

**Joan C. Marini, M.D., Ph.D., Chief**

Investigators in the Heritable Disorders Branch (HDB) seek to understand the biochemical and molecular mechanisms of heritable disorders and to apply this information to the treatment of affected children.

*Joan Marini's* group focuses on osteogenesis imperfecta, also known as brittle bone disease. The group is engaged in parallel treatment trials of bisphosphonate drugs in mice and children with OI. The murine study uses the Brtl mouse model for OI that was developed in the HDB; the model will allow direct determination of physical properties and biomechanics of OI bone. The laboratory has provided the first quantitation of the burden of mutant osteoblasts that is compatible with normal skeletal functioning in OI mosaics. The resultant information sets the target levels for cell therapy of dominant disorders of the skeleton at 50 to 60 percent

*Forbes Porter's* group studies inborn errors of cholesterol synthesis, including Smith-Lemli-Opitz syndrome (SLOS). Using gene targeting in murine embryonic stem cells, the research team has produced three SLOS mouse models, including a null mutation and two hypomorphic point mutations. The null pups have the biochemical features of SLOS and decreased glutamate response consistent with neurological dysfunction in SLOS, but they die on the first day of life. The hypomorphic mouse mutants are non-lethal and will allow studies of pathophysiology and potential therapeutic interventions. The research group has provided insight into the autistic and obsessive-compulsive traits of SLOS patients and elucidated the common occurrence of compensated adrenal insufficiency.

*Janice Chou's* group investigates glycogen storage disorders, developing approaches to somatic gene therapy that target the integral membrane–associated G6Pase enzyme to the liver and kidney. Treatment of the GSD-Ia mice generated in this laboratory with adeno- and adeno-associated virus vector–mediated gene transfer corrects the murine disease for a year and provides a model that might be applied to patients to eliminate long-term disease complications. Investigations of GSD-Ib, caused by defects in G6P transporter, have focused on mutation analysis. Most mutations completely abolish the transporter, but about a third have residual activity.

*Anil Mukherjee's* group focuses on several genes, including palmitoyl-protein thioesterase and neutral ceramidase. Inactivating mutations in the PPT gene cause infantile neuronal ceroid lipofuscinosis (INCL), a severe neurodegenerative disease of childhood with no effective treatment. Using a Bench to Bedside Award, the group has initiated a pilot study to determine whether Cystagon is beneficial for INCL patients. Cystagon has virtually eliminated the ceroid deposition in white blood cell lysosomes of these patients, and an evaluation of the effect of treatment on neural degeneration of affected children is in progress.

A group led by *Ida Owens* investigates the mammalian UDP-glucuronosyltransferase (UGT) isozymes, which are critical for detoxifying endogenous and ingested toxins by conjugation with glucuronic acid. The research team has demonstrated that transient inhibition of gastrointestinal UGT activity markedly improves mycophenolic acid immunosuppressant activity. The team's work has yielded a potential model for increasing the bioavailability of highly glucuronidated drugs. Studies combining *in situ* and biochemical analyses found that the enzymes were segmentally distributed throughout the GI in mucosal epithelia.

## LABORATORY OF CELLULAR AND MOLECULAR BIOPHYSICS

**Joshua Zimmerberg, M.D., Ph.D., Chief**

The laboratory has continued to shed light on the essential mechanisms of life processes, both at the physico-chemical level and the level of human tissues and diseases. *Joshua Zimmerberg's* group tested whether lipid pores and hence intrinsic membrane curvature mediates programmed cell death (apoptosis). Consistent with their model, apoptotic pores were indeed sensitive to lipid curvature. A new large-scale method to culture the major malaria parasite gave high yields of parasitized erythrocytes (and potentially immunogens thereof) both cost-effectively and with reduced labor. Parasites replicated at the packed densities found in brain microvasculature in fatal cerebral malaria. Novel immunoblotting techniques for quantitative analysis of the SNARE protein complement of functional secretory vesicles achieved routine determination in the femtomole to attomole range. The group proposed a microdomain in a ring topology (ring rafts) as a barrier to lipid diffusion to facilitate fission.

Influenza viral infection starts with viral entry via membrane fusion. Work in *Leonid Chernomordik's* group showed positive inter-trimer cooperativity in the triggering of the conformational change in fusion proteins; HA refolding that both involves reversible conformations with a lifetime of minutes, and plays an important role in coupling between this refolding and fusion, allowing adjacent trigger-activated proteins to assemble at the contact site where concerted discharge of the conformational energy drives fusion; and an interconnected coat that is formed by membrane-bound activated fusion proteins surrounding the membrane contact zone and may generate the driving force for fusion, bending out of the initial shape of the membrane surface and producing elastic stresses in the underlying lipid bilayer.

Critical events of HIV disease occur in lymphoid tissue. *Leonid Margolis's* group found that CXCR4-tropic HIV-1 suppresses replication of CCR5-tropic HIV-1 in human lymphoid tissue *ex vivo* by selective induction of CC-chemokines and that X4 and R5 HIV-1 variants interfere with each other, with the X4 inhibition of R5 replication possibly explaining a shift seen with clinically worsening cases of AIDS; that dramatic suppression of CCR5, but not CXCR4-HIV-1 replication, occurs in tissues coinfected with herpes virus HHV-6; that, in tissues infected with HHV-6, production of RANTES increases, with the addition of RANTES causing inhibition of R5, but not X4 replication; and that HHV-6 replication, an event frequently occurring *in vivo* during the progression of HIV-1 disease, selectively suppresses CCR5- HIV-1 variants.

*Al Yergey's* group is measuring energies required for the formation of non-covalent bonds between molecules and ions in solution, particularly those involving water and biologically significant molecules. He also established a facility to identify and characterize proteins and peptides at a microscale level. Three mass-spectrometric approaches are available: MALDI with TOF mass analysis, LC-MS/MS, MALDI–TOF/TOF. He is developing ways to identify multiple phosphorylations on single peptides with the MALDI TOF/TOF. In the area of protein separation, *Andreas Chrambach* made available on the Internet (http://www.ncbi.nlm.nih.gov/Class/wheeler/jovin.html) buffer systems for enhancing gel electrophoresis resolution and developed a three-step procedure making membrane proteins susceptible to proteomic analysis.

## LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY

**Mark L. Mayer, Ph.D., Chief**

Studies in the Laboratory of Cellular and Molecular Neurophysiology focus on the receptors and ion channels that mediate signalling in the central nervous system. Excitability in the brain takes many forms. On the millisecond time scale, cells fire action potentials and communicate via activation of ligand-gated ion channels at excitatory and inhibitory synapses. Intimately linked to signalling on this fast scale are second messenger systems that control a wide range of metabolic processes. Ultimately, changes in activity mediated by a vast network of electrical and biochemical processes are tied to the regulation of gene expression itself. Such regulation underlies our creative abilities to rationalize thoughts, experience emotions, and recall memories. Our experiments target glutamate receptor ion channels, the molecules mediating the initial response at excitatory synapses. The atomic resolution of ion channel structure made possible by crystallography and x-ray diffraction, combined with results from physiological and biochemical approaches, has made it possible for the first time to propose molecular mechanisms underlying activation and desensitization for glutamate receptor ion channels. By extending this approach, we hope to gain insight into the molecular basis for the behavior of individual glutamate receptor subtypes.

## LABORATORY OF CELLULAR AND SYNAPTIC NEUROPHYSIOLOGY

**Chris McBain, Ph.D., Chief**

The Laboratory of Cellular and Synaptic Neurophysiology uses the techniques of neurophysiology, molecular biology, and cell biology to study signalling mechanisms related to the development, physiology, and pathophysiology of the mammalian central nervous system. Specifically, we study receptors, ion channels, and signalling mechanisms in preparations that range from isolated cells to highly ordered neural networks in physiological and pathophysiological conditions in both wild-type and transgenic animals. Problems under analysis concern mechanisms of short- and long-term plasticity of synaptic transmission, neurotrophin regulation of excitability and development, differential targeting of synaptic receptors, neuron-glial signalling, pathophysiological processes in clinically relevant neuronal migration disorders, ion channel regulation of development and excitability, and drug action at a variety of voltage- and ligand-gated receptors.

During the last year, *Chris McBain* conducted an extensive characterization of $Ca^{2+}$-permeable, GluR2-lacking AMPA-, and NMDA-receptors at hippocampal inhibitory interneuron synapses. His group elucidated the time course of electrotonically overlapping but kinetically distinct synaptic conductances on inhibitory interneuron dendrites and the roles of voltage-gated conductances in the generation of muscarinic receptor– and kainate receptor–induced hippocampal gamma oscillatory activity. *Doug Fields* elucidated the role of adenosine as a neuron-glial transmitter that promotes myelination in the central nervous system in response to action potential activity. He also studied the role of electrical activity in regulating late-phase long-lasting synaptic plasticity. *Bai Lu* worked on activity- and tyrosine kinase–dependent facilitation of trkB receptor internalization in hippocampal neurons and discovered the presence of single nucleotide polymorphisms in the BDNF gene and the role played by the polymorphisms in BDNF secretion and hippocampal function, with particular reference to memory formation. *James Russell* investigated the kinetics of $Ca^{2+}$ release from $Ca^{2+}$-signalling microdomains and the pattern of type 2 $IP_3$Rs expressed by astrocytes *in situ*.

Both Drs. Fields and Lu gained tenure. Dr. McBain was elected to the editorial board of the *Journal of Physiology* and *Journal of Neuroscience*. Finally, the laboratory made considerable effort to recruit two independent researchers to the NICHD tenure track: in fall 2002, Drs. Dax Hoffman and Mark Stopfer joined the LCSN. *Dax Hoffman* combines patch clamp recording in brain slices of hippocampus with molecular biology techniques to investigate the electrophysiological properties and molecular nature of the voltage-gated channels expressed in CA1 dendrites. *Mark Stopfer* combines imaging, patch clamp physiology, and behavioral analysis to investigate synaptic and network mechanisms underlying sensory processing and memory formation, including coding mechanisms involving the oscillatory interactions of ensembles of interneurons in the insect antennal lobe.

## LABORATORY OF CLINICAL GENOMICS

**Owen M. Rennert, M.D., Chief**

The Laboratory of Clinical Genomics (LCG) consists of two laboratories and the Unit on Pediatric Genetics and the Section on Developmental Genomics. Investigators within the LCG have initiated three new clinical studies. Under the direction of lead investigator *Margarita Raygada*, the first focuses on the natural history of inborn errors of metabolism and birth defects, specifically those associated with deficits in central nervous function. The second study, just initiated, is investigating gene-modifiers, specifically insulin, IGF, and related growth factors, in patients with breast cancer while the third study, also just initiated, is exploring the genetic consequences of the *BSCL2* locus (chromosome 11Q13) defect in Berardinelli congenital lipodystrohy.

*Stephen Kaler* heads the Unit on Pediatric Genetics, which has focused on inborn errors of trace metal metabolism, specifically copper metabolism and the copper-transport disorder Menkes' kinky hair disease. Dr. Kaler is also investigating the causes of the human platelet disorder Bernard-Soulier syndrome and the X chromosome–linked Rett syndrome and the related phenotype PHACE.

The Section on Developmental Genomics, led by *Owen Rennert*, has focused on identifying the network of genes expressed in germ cells at four different stages of spermatogenesis, namely, gonocyte, type A spermatogonia, pachytene spermatocyte, and round spermatid. The laboratory achieved the identification of differentially expressed genes by using cDNA microarray hybridization and mapping the transcriptome with Serial Analysis of Gene Expression (SAGE). The research team identified 240 differentially expressed genes, more than one-quarter of which were previously uncharacterized. Genes responsible for signal transduction, energy metabolism, biosynthesis, and cellular transport were preferentially expressed in spermatids while genes for chromatin remodeling were expressed only in spermatocytes. Analyses of SAGE libraries allowed identification of more than 23,300 genes, among which 699 were specific for type A spermatogonia, 3,199 were specific for pachytene spermatocytes, and 2,836 were specific for round spermatids. More than 2,500 of these genes were novel. This number of germ cell genes is far greater than that contained in any known database and constitutes a rich source of information for future studies on genetic regulation of spermatogenesis.

Members of the section have continued their investigation of the molecular genetics of the luteinizing hormone/gonadotropin receptor (LHR) in human diseases. They identified a homozygous Leu502Pro substitution in transmembrane helix (TM) IV of the LHR, which causes a severe form of Leydig cell hypoplasia. This substitution probably disrupts the $\alpha$ helical structure of TM IV, resulting in inactivation of LHR. It is the first disease-causing mutation identified in TM IV of LHR, suggesting that TM IV plays an important role in the active-inactive transition of the receptor.

A new project was initiated to identify genetic risk factors for the development of pseudotumor cerebri (PTC). Although the majority of cases are non-familial, reports of familial PTC have suggested the possibility of some genetic variation/defect that may become clinically apparent after exposure to a precipitating agent. Studies of a Turkish cohort of PTC patients resulted in identification of a single nucleotide substitution in exon 10 of the coagulation Factor V, which was significantly associated with the development of PTC. This is the first study to document a genetic risk factor for PTC.

## LABORATORY OF COMPARATIVE ETHOLOGY

**Stephen Suomi, Ph.D., Chief**

The Laboratory of Comparative Ethology carries out a program of basic biobehavioral research that investigates cognitive, social-emotional, and biological development in humans and nonhuman primates. The laboratory studies both genetic and environmental factors and their multiple interactions from a comparative perspective in order to characterize the developmental trajectories of individuals across a broad range of species, populations, and settings. Field and laboratory-based studies of biobehavioral development in nonhuman primates are designed specifically to facilitate comparisons with findings from long-term prospective investigations of human infants and their families as well as with data obtained by neuroscience techniques, thereby promoting translational analyses. Most studies employ longitudinal designs in order to address basic issues of continuity versus change and the relative stability of individual differences throughout development. Many investigations concomitantly collect a variety of both behavioral and biological measures reflecting multiple levels of analysis, thus making possible the examination of patterns of covariance among measures both within and between levels of analysis. To identify particularly recurrent or unusual patterns and evaluate their consequences with respect to established norms, the laboratory places major emphasis on characterizing and understanding normative patterns of development.

*Stephen Suomi* and his colleagues investigate biological and behavioral development in selected nonhuman primate species, focusing on interactions between genetic and environmental factors that affect the course of individual developmental trajectories throughout the life span. *John Newman* uses neuroscience techniques to study brain mechanisms involved in the production of various types of primate vocalizations, to examine subtle acoustical differences in these characteristic calls, and to investigate their function in several primate species. *Marc Bornstein's* group examines cognitive, language, and social-emotional development in human infants and children, with special emphasis on the relationships among early attentional processes, social stimulation from and interactions with care givers, and subsequent cognitive and social behavioral capabilities. *Michael Lamb* heads a team that studies the effects of different types of care-giving practices on human infant and toddler social and emotional development and cognitive competence, with particular emphasis on process-oriented studies of interactional styles, memories, and social perceptions and longitudinal approaches that transcend infancy, childhood, and adolescence.

## LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY

**John B. Robbins, M.D., Chief**

The Laboratory of Developmental and Molecular Immunity (LDMI) studies the pathogenesis of and immunity to bacterial diseases, especially those of infants and children, with the overall objective of developing vaccines for their prevention. Given that almost all pathogens of healthy individuals are inhabitants of humans only, the LDMI brings its investigational vaccines to clinical evaluation for safety, immunogenicity, and, ultimately, efficacy, as soon as possible. Research at LDMI is guided by the principle that "the proper study of mankind is man."

Members of the LDMI pioneered the development of a new generation of vaccines in which specific antigenic capsular polysaccharides are chemically conjugated to highly immunogenic but nonspecific proteins. The new conjugate vaccines, which confer T-cell dependence and booster responses to polysaccharide antigens, have been singularly successful, as exemplified by the *H influenzae* type b conjugate vaccine. *H. influenzae* type b meningitis (the most common cause of acquired mental retardation) has been virtually eliminated wherever the vaccine has been used. The principles and methods developed for this vaccine have now yielded conjugate vaccines against *Salmonella typhi*, nontyphoidal *Salmonellae*, *Shigellae*, and *Vibrio cholerae*, which are being field tested. By producing a nontoxic mutant shigella-like toxin and conjugating it to the capsular polysaccharide of *E. coli* 0157, LDMI researchers produced an experimental vaccine against this pathogen, which causes the often fatal hemolytic uremic syndrome, especially in small children. A conjugate vaccine against *Staphylococcus aureus,* the cause of many hospital-acquired infections, has been tested successfully in hemodialysis patients, who are also at increased risk of infection by this pathogen. In an effort to produce an anthrax vaccine with fewer side effects than the currently available vaccine, a recombinant vaccine against *Bacillus anthracis*, produced on an industrial scale in collaboration with researchers at NIDDK, has been shown, in mice, to elicit levels of neutralizing antibody comparable to those elicited by the currently available vaccine.

## LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

**Y. Peng Loh., Ph.D., Acting Chief**

The goal of the Laboratory of Developmental Neurobiology is to understand the molecular and cellular mechanisms underlying the regulation of development and function of the nervous system. Major topics of interest include the mechanisms of processing, intracellular trafficking, and secretion of neuropeptides and hormones; the molecular machinery of secretory vesicle biogenesis and the presynaptic release process; circadian biology with emphasis on the pineal gland and synthesis of melatonin; the coupling between action potentials, neurotransmitters, neurohormones, neurotrophic factors, and the regulation of gene expression. The laboratory carries out its investigations with a variety of cellular systems as well as with knock-out and transgenic animal models. Researchers use wide-ranging analytical techniques that include gene chips, imaging, immunohistochemistry, electron microscopy, and behavioral testing to examine neuronal cell biology, activity-dependent neural and neuromuscular plasticity, and the effects of neurotrophic factors on fetal nervous system development.

Advances include work from *Peng Loh's* group, which discovered a master molecule, chromogranin A, that regulates secretory granule biogenesis at the transcriptional and post-translational level and hence neurotransmitter secretion from neuroendocrine cells. Her group has also elucidated the molecular basis for the intracellular missorting of mutant proinsulins in hyperproinsulinemia patients that leads to diabetes. *David Klein's* group has established an important element, the binding protein 14-3-3, that regulates the level of the enzyme serotonin N-acetyltransferase that is responsible for the melatonin rhythm-generating system. *Andres Buonanno's* group has discovered a new transcription factor, General Transcription Factor 3 (GTF3), that is expressed in numerous tissues, including muscle and brain, and contributes to the establishment of fiber types during perinatal development. The genes encoding GTF3 and its related homolog GTF2i are deleted in individuals with Williams syndrome (WS) who manifest numerous cognitive deficits as well as myotonias. The group's recent studies using ectopically transfected GTF3 constructs in adult muscles and GFT3-knock-out mice support a role for GTF3 in regulating muscle contractile properties that could be related to the myopathies observed in WS. *Phillip Nelson's* group has shown that the ras-mitogen–activated protein kinase (MAPK) and cAMP/PKA-mediated pathways play important roles in neuronal plasticity and Hebbian synapse modulation at the neuromuscular junction. *Douglas Brenneman's* group has characterized ADNF, a glial-derived factor involved in the control of neuronal survival, as a multi-component protein complex that include several proteases. Work from *Elaine Neale's* group has shown that part of the lethal effect of botulinum toxin A, considered a potential agent of terrorism, may be attributable to the severe retardation of the rate of replenishment of synaptic vesicles in the readily releasable pool at the nerve terminal, leading to a failure in neurotransmission.

## LABORATORY OF GENE REGULATION AND DEVELOPMENT

**Alan G. Hinnebusch, Ph.D., Chief**

Five of the eight groups in the Laboratory of Gene Regulation and Development study gene regulation and chromosome structure in the unicellular eukaryotes *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Having shown previously that four eukaryotic translation initiation factors (eIFs 1, 2, 3, and 5) reside in a multifactor complex with an important function in translation initiation, the section headed by *Alan Hinnebusch* recently identified independent contacts between eIF2 and eIF3 in the multifactor complex that make additive contributions to a critical step in the pathway downstream from 48S complex assembly. The researchers also showed that the kinase GCN2, which regulates translation by phosphorylating eIF2, is controlled by the TOR signalling pathway. Another section of the laboratory, headed by *Thomas Dever*, is characterizing several eIFs and the protein kinases that regulate eIF2 activity, focusing on principles of kinase-substrate recognition. The team determined that GTP binding induces structural rearrangements in the universally conserved translation initiation factor eIF5B/IF2, which regulates the factor's affinity for the ribosome.

Gene silencing in yeast is the focus of a unit, headed by *Rohinton Kamakaka,* that characterizes Sir2 protein complexes and recently demonstrated a role for histone Htz1 in cell-cycle progression and silencing. The unit also identified proteins involved in chromatin remodeling that have the ability to impede the spread of silenced chromatin. Another unit, headed by *Alexander Strunnikov,* is investigating the molecular mechanisms of chromosome condensation (condensin complex) and sister chromatid cohesion (cohesin complex). The group recently dissected the role of post-translational modifications in the regulation of condensin and reconstituted the cohesin-chromatin interaction *in vitro* and began dissecting the molecular nature of SMC complexes in bacteria.

The section headed by *Henry Levin* is analyzing a retrotransposon that is structurally related to mammalian retroviruses. The section recently identified residues in reverse transcriptase that are required for removal of the RNA primer from the completed cDNA as a prerequisite for integration and also identified two host factors required for Tf1 transposition, a methyltransferase that methylates lysine 4 of histone H3 and a factor that mediates vesicle traffic.

The newly formed unit headed by *Chi-Hon Lee* investigates the molecular basis of connection specificity in the *Drosophila* visual system, focusing on N-cadherin function. The group recently discovered that N-cadherin loci undergo alternative splicing to generate multiple isoforms and suggested that combinatory use of these isoforms provides a "synaptic code" for matching pre- and post-synaptic partners.

The anuran *Xenopus laevis* serves as a model system for the remaining two groups. The unit headed by *Yun-Bo Shi* studies the role of thyroid hormone (TH) and its receptor (TR) during metamorphosis and the functions of matrix metalloproteinases (MMPs) induced by TR in organ remodeling. The group recently demonstrated that unliganded TR recruits to TH-response gene corepressors that in turn recruit histone deacetylases in order to repress gene expression in premetamorphic tadpoles, that transgenic overexpression of a dominant negative TR blocks the expression of TH-response genes and metamorphosis, and that the MMP stromelysin-3 is required for TH-induced apoptosis in the intestine. *Mary Dasso's* group studies the small ubiquitin-like protein SUMO-1 and recently showed that SUMO-1 conjugation and deconjugation enzymes localize to opposite sides of the nuclear pore in mammals. The researchers also demonstrated that SUMO-1 conjugation regulates the mitotic localization of the GTPase-activating protein (RanGAP1) for Ran, a GTPase that is essential for nuclear transport in all eukaryotes.

## LABORATORY OF INTEGRATIVE AND MEDICAL BIOPHYSICS

**Ralph Nossal, Ph.D., Chief**

The Laboratory of Integrative and Medical Biophysics (LIMB) performs cross-disciplinary research leading to a deeper understanding of cell and tissue processes in both normal and disease states. It also develops new methodologies for biomedical research and diagnosis. The laboratory's work joins biomedical research to experimental and theoretical techniques commonly associated with research in the physical and engineering sciences. The staff's specialized interests and expertise include optical imaging of biological tissues, magnetic resonance imaging, mathematical modeling, methods of quantitative cell biology, techniques for assessing ultra-small biological samples, and polymer physics and physical chemistry. The laboratory's unique capabilities extend to advanced physical methods (e.g., photon and neutron scattering, magnetic resonance and optical imaging) and an ability to formulate mathematical and computational models. Investigators study biological function at various levels of complexity, from molecular to tissue, focusing on interactive behavior at different length and time scales. Much of LIMB's work is strongly collaborative, and a number of research projects are carried out with colleagues in other NIH branches and laboratories as well as with investigators at other institutions.

LIMB is organized into four areas that reflect its several interests. The Section on Tissue Biophysics and Biomimetics, under *Peter Passer*, works to gain a better understanding of tissue-level processes, such as nerve excitability and load bearing in cartilage, by developing biomimetic tissue analogs and new physical theories and computational models to aid in the design and interpretation of biological experiments. The group is also continuing its development of Diffusion Tensor Magnetic Resonance Imaging (DT-MRI) as a probe of tissue structure in normal or diseased organs, with particular emphasis on neurological tissues. The Section on Medical Biophysics, under *Robert Bonner,* develops new optically based biophysical methodologies for research and clinical applications. Continuing work focuses on advancing Laser Capture Microdissection and related technologies as tools for isolating targeted cells for use in both pathological investigation and studies of developing organisms. In addition, this group is developing analytic and statistical techniques for quantitative analysis of gene expression. The Section on Cell Biophysics, under *Ralph Nossal,* aims to understand the physical basis for various cell activities that involve structural changes in supramolecular biomolecular complexes. Currently, its principal focus is on elucidating processes underlying the formation of vesicles involved in intracellular trafficking and understanding the formation and transformation of supramolecular tubulin structures involved in various critical cell activities. The general long-term goal is to build and use an arsenal of tools, both theoretical and experimental, to study kinetic aspects of cell processes and to increase knowledge of the ways those activities can be mediated by external interventions. The Unit on Biomedical Stochastic Physics, under *Amir Gandjbakhche*, works on interactions between light and tissue as related to non-invasive optical imaging of biological targets. The unit's long-term objective is to develop novel approaches to tomographic imaging and quantitative optical spectroscopy of deeply buried tissue structures, including tumors. To that end, the unit is carrying out a multifaceted experimental and computational research program that incorporates mathematical and physical theories and technologies, experimental models, and collaborative clinical investigations.

## LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

**Heiner Westphal, M.D., Chief**

The Laboratory of Mammalian Genes and Development generates gene-altered mice to study pattern formation, T cell development, and genomic imprinting. The mutant mice also serve as paradigms for human genetic disorders. The laboratory comprises three independent research groups.

The Section on Mammalian Molecular Genetics, headed by *Heiner Westphal*, studies genes that regulate embryonic development. Currently, the section is focusing on LIM-domain–containing regulators of development and cofactors that bind to them. Details have emerged during the past year on two cofactors, termed Ldb1 and Ssdp. These gene products are essential for embryonic development and have been well preserved throughout invertebrate and vertebrate evolution. Gain- and loss-of-function studies in the mouse, *Xenopus*, and *Drosophila* have shown that Ldb1 and Ssdp mediate the action of a number of LIM factors that exert important controls during gastrulation and organogenesis.

The Section on Cellular and Developmental Biology, directed by *Paul Love*, focuses on the molecular genetics of T cell development, with special emphasis on genes that regulate signal transduction in developing and mature T cells. Recent studies revealed a critical role for the lymphocyte adaptor protein LAT in transmitting signals from the cell surface to downstream pathways that control T cell maturation and function. These experiments also demonstrated that coordination of lymphocyte signalling responses is essential to prevent abnormal cell proliferation. All mammals contain two lineages of T cells, alpha/beta and gamma/delta, that are distinguished by the clonotype-specific chains in their T cell antigen receptors (TCRs). In another study, members of the laboratory demonstrated a fundamental difference in the subunit composition of the signal-transducing complexes of the alpha/beta and gamma/delta TCRs; a difference in TCR structure may influence the activation kinetics and functional responses of these distinct T cell lineages. A third area of investigation centered on studying the molecules that control T cell migration and trafficking, demonstrating a role for the chemokine receptor CCR9 in regulating homing of T cell progenitors to the thymus and their migration within the thymus during development.

The Unit on Genomic Imprinting, led by *Karl Pfeifer*, works on the regulated expression and biological function of a cluster of genes on the distal end of mouse chromosome 7. These genes share an unusual form of gene regulation, genomic imprinting, that is specific to mammalian organisms. Imprinted genes are expressed from only one chromosome in a parent-of-origin–dependent manner. The research group has focused on identifying the epigenetic markers that distinguish maternal and paternal chromosomes and on understanding the mechanisms by which these markers result in monoallelic gene expression. The organization and regulated expression of these genes is well conserved in humans, and the syntenic region is associated with Beckwith Wiedemann syndrome, a developmental disorder, as well as with inherited cardiac arrhythmias. The unit has successfully established a mouse model for this heart condition and is using the mouse to understand how b-adrenergic signals might stimulate cardiac malfunction in humans.

## LABORATORY OF MOLECULAR GENETICS

**Igor B. Dawid, Ph.D., Chief**

The Laboratory of Molecular Genetics (LMG) pursues studies in different systems that aim to elucidate molecular-genetic mechanisms of physiological and developmental control, in particular the regulation of signalling and signal transduction cascades during development. Of note are the discoveries made by the groups of two tenure-track investigators, both working on the zebrafish, a premier vertebrate model system for the study of embryogenesis.

The group of *Brant Weinstein* works on vascular biology, specifically the mechanisms of vasculogenesis, the initial generation of blood vessels in the embryo, and the molecular-genetic distinctions that define arterial and venous identity. While functional redirection of veins to arteries and *vice versa* can take place in the organism, recent work completed by many groups, including this LMG group, indicates that there is a genetically programmed distinction between different vessels when they first arise in the embryo. Work by Dr. Weinstein and colleagues has illuminated the signalling cascade that defines arterial identity in the embryo. By using a combination of genetic, molecular, and embryological techniques, the researchers showed that vascular endothelial growth factor (VEGF) plays a major role in embryonic arterial development. VEGF is expressed under the control of hedgehog signalling in the embryo and, in turn, regulates the activity of Notch that gives the proximal signal leading to vessels with arterial identity.

Identification of the contribution of Notch signalling to vessel formation relied in part on the use of a mutation, *mindbomb (mib)*, in which too many primary neurons are generated in the embryo as a result of deficits in the Notch signalling cascade. *Ajay Chitnis's* group discovered and has studied this mutant. Notch is a signalling system of major importance in many developmental systems and plays a role in some human tumors. Dr. Chitnis and his colleagues have shown that the *mib* mutation disrupts a gene that encodes a so-called E3 ligase. E3 ligases are the specificity-endowing components of the ubiquitin-dependent protein modification and degradation machinery, and the *mib* gene product functions specifically in the Notch pathway. Chitnis and colleagues' work represents a major advance in expanding our understanding of the important and much-studied Notch pathway and, as such, has far-reaching implications in biology.

## LABORATORY OF MOLECULAR GROWTH REGULATION

**Bruce H. Howard, M.D., Chief**

The Laboratory of Molecular Growth Regulation conducts research on the control of cell proliferation, DNA replication, and gene regulation in eukaryotic cells. With respect to the last, independent groups work in complementary areas, including gene regulation of the developing immune system, control of gene expression during early embryogenesis, chromatin-mediated gene silencing, and transcription of small RNA-encoding genes. Of particular interest with respect to immune system function are transcription factors that control cell proliferation and differentiation. Studies by *Keiko Ozato* and her coworkers on ICSBP/IRF-8 have revealed that this transcription factor is essential for the proper development of macrophages, granulocytes, and dendritic cells. Defects in these cell lineages can be rescued by introduction of the ICSBP gene into cells derived from knock-out mice. Recent work has focused on the identification of critical target genes controlled by ICSBP. One such target gene, IL12-p40, is a cytokine that plays an essential role in the development of innate immunity. Microarray studies have revealed at least 50 additional gene products whose expression is up- or down-regulated by ICSBP. Ozato and her colleagues have shown that both ICSBP and Brd4, another transcription factor under investigation, function in part by regulating the activity of chromatin. *Melvin DePamphilis* is leading a series of investigations into gene regulation during early mouse embryogenesis involving the transcription factor mTEAD-2. Interestingly, a number of promoters and enhancers become responsive to mTEAD-2 concomitant with the onset of zygotic gene expression. Recent studies have led to the identification of YAP65 as a long sought-after co-activator of the TEAD family of transcription factors. YAP65, which also binds to Src/Yes protein kinases and interacts with the cytoplasmic localization protein 14-3-3, is a strong candidate for the transducer of mitogenic signals that activate TEAD-dependent transcription. *Richard Maraia* directs studies focusing on the multifunctional factor known as human La antigen. La has been characterized as a regulatory chaperone for nascent RNAs. Work in this laboratory has been instrumental in defining the precise roles of La in the nuclear trafficking and processing of associated tRNAs. Related studies have highlighted the regulatory function of a major phosphorylation site in the La antigen. In this case, phosphorylation modulates the interaction of La with nascent transcripts as well as its trafficking within the nucleus. Given that these studies have revealed that the protein contains an efficient carrier-mediated nuclear export signal, the chaperone function of La appears also to include export to the cytoplasm. Finally, a major area of interest for the laboratory concerns higher-order chromatin structure, in particular how defects in the maintenance of such structures may underlie developmental disorders and age-related diseases. Under the direction of *Bruce Howard*, the researchers have developed random sampling approaches to facilitate the mapping of chromatin structures across the genome and to enable them to detect areas of chromatin remodeling. In related work, they have linked a form of chromatin-mediated gene expression control, termed imprinting, to histone H3 methylation.

## LABORATORY OF PHYSICAL AND STRUCTURAL BIOLOGY

**V. Adrian Parsegian, Ph.D., Chief**

The Laboratory of Physical and Structural Biology is staffed largely by physicists who take an integrative approach to the disciplines of physics, biology, and chemistry. They see the next step in structural biology not simply as determining the structures of every identifiable entity from molecule to organelle but rather as learning how these structures work through the physics and chemistry of the intermolecular forces that drive them. Only then can we learn from the increasing number of protein, nucleic acid, saccharide, and lipid structures how to design agents that compete effectively with deviant interactions associated with disease. It is gratifying to see that the physics they have developed, which explains bio-matter, is finding applications outside biology.

By reconstituting an OmpF into lipid bilayers and monitoring interference with channel conductance, members of the laboratory, led by *Sergey Bezrukov*, have been able, during the past year, to mimic the passage of an antibiotic such as ampicillin through a bacterial trans-membrane ionic channel. One remarkable lesson from this observation is that weak binding of ampicillin, as revealed by extended residence times, improves the antibiotic's chances of making it all the way through to the other side.

*Don Rau's* group has been able to show how solution conditions modify the specific versus non-specific binding of proteins to DNA. Thus, under osmotic stress, DNA/protein association involves about 100 more intervening water molecules in non-specific than in specific association; the difference is seen in association constants as well as in rates of dissociation.

*Adrian Parsegian's* group has formulated the strength and range of forces that stabilize membranes used in drug-delivery systems. Created by polymers bound to lipid bilayer membranes, these forces have been previously measured and are now correlated with the osmotic pressures of the same polymers in solution. Using the correlation, the group can now design polymer-stabilized systems more systematically than was formerly possible.

## PEDIATRIC AND REPRODUCTIVE ENDOCRINOLOGY BRANCH

**George P. Chrousos, MD, Chief**

The Pediatric and Reproductive Endocrinology Branch (PREB) focuses on the physiology and pathophysiology of growth, development, metabolic, immune, and reproductive functions and the major neurohormonal systems, the hypothalamic-pituitary-adrenal and -gonadal axes and autonomic nervous system, that subserve these functions. The researchers study both the developmental and static functions of these systems and their key effector molecules, including corticotropin-releasing hormone, arginine-vasopressin, corticotropin, glucocorticoids, gonadotropins, estrogens, progestins, androgens, and the catecholamines, in the *in vivo* integrated and at the reduced cellular, subcellular, and genomic levels. The branch oversees operation of the NICHD Component of the Inter-Institute Endocrinology, Metabolism and Diabetes Program, directed by *Lynnette Nieman*, and the Reproductive Endocrinology and Infertility Training Program, which have earned the approval, respectively, of the Accreditation Council for Graduate Medical Education (ACGME) and the American Board of Obstetrics and Gynecology. The branch also participates in the ACGME-accredited Pediatric Inter-Institute Endocrinology and Metabolism Program.

Led by *George Chrousos*, the Section on Pediatric Endocrinology demonstrated novel mechanisms leading to sporadic, familial, inflammation-related and physiologic glucocorticoid resistance and described a novel molecular mechanism of glucocorticoid resistance in response to inflammatory cytokines and of HIV-1-induced glucocorticoid hypersensitivity, potentially a major pathogenetic factor in the development of AIDS. The researchers also demonstrated that glucocorticoids influence approximately one-fifth of the human genome, including a wide array of genes of the immune response, and described a mechanism whereby glucocorticoids and catecholamines cause the shift from cellular to humoral immunity. Other findings include epinephrine deficiency and insulin resistance, along with inability to elevate glucose levels in response to exercise in children with congenital adrenal hyperplasia. The group demonstrated major changes of HPA axis and immune system functions in normal individuals exposed to acute or chronic sleep loss, in patients with idiopathic chronic insomnia, and in patients with sleep apnea, showing that sleep apnea affects many patients with polycystic ovary syndrome and is part of the metabolic syndrome X.

Under *Lynette Nieman,* the Section on Reproductive Medicine, together with Dr. Pamela Stratton, head of the Ob/Gyn consultation service, demonstrated that endometriosis had marked comorbidity with the autoimmune disorders fibromyalgia and chronic fatigue syndrome as well as with infertility. The researchers also showed that MRI had poor predictive ability for surgery-proven endometriosis.

Led by *Karel Pacak,* the Unit on Clinical Neuroendocrinology is investigating the etiology, pathophysiology, diagnosis, prognosis, and treatment of pheochromocytoma and other neuroendocrine tumors. The group introduced plasma-free metanephrine in the diagnosis of pheochromocytoma and PET scanning in the localization of pheochromocytoma tumors, described major differences between such tumors when associated with different multiple neoplasia syndromes, and established molecular markers of benign and malignant pheochromocytoma.

The Unit on Reproductive Endocrinology and Infertility investigated a variety of clinical reproductive disorders. The studies were an integral facet of the Reproductive Endocrinology and Infertility Training Program, directed by *James Segars*. The research team identified a number of genes specifically associated with fibroids; those genes are now undergoing analysis for clues in uterine muscle tumorigenesis. The team also explored the relation of the Brx protooncoprotein, which it cloned, to estrogen-mediated responses, implicating p38 mitogen–activated protein kinases in estrogen action.

## PERINATOLOGY RESEARCH BRANCH

**Roberto Romero, M.D., Chief**

The Perinatology Research Branch conducts clinical and laboratory research in maternal and fetal diseases responsible for excessive infant mortality in the United States. The branch focuses on the study of the mechanisms of disease responsible for premature labor and delivery, with particular emphasis on the role of subclinical intrauterine infection. The prenatal diagnosis of congenital anomalies is also a major area of interest.

This year, NICHD awarded a major contract and lease to establish a facility and support the operations of the Perinatology Research Branch in the state of Michigan. The new campus offers access to a patient population with a high rate of pregnancy complications and brings to bear the academic resources of three major universities: Wayne State University, University of Michigan, and Michigan State University. The branch plans to develop strong collaborative relationships with these universities and the Van Andel Research Institute. These organizations are part of the Life Science Corridor established by the state of Michigan to attract and support research and development in the biomedical field.

Premature birth is the leading cause of perinatal mortality and morbidity worldwide. The Perinatology Research Branch has defined preterm labor as a syndrome and determined that at least 25 percent of all preterm infants are born to women with sub-clinical intrauterine infection. Moreover, the branch has provided evidence that a substantial number of premature neonates are critically ill before birth; thus, it has proposed that the onset of premature labor has survival value in the context of intrauterine infection. This year, the branch conducted a number of studies focusing on the mechanisms responsible for preterm premature rupture of membranes (PROM), which accounts for 40 percent of all preterm births. The branch has accumulated substantial evidence that matrix-degrading enzymes, such as matrix-metalloproteinase (MMP)-1 (interstitial collagenase), MMP-9, neutrophil elastase, and collagenase, are involved in the mechanism of membrane rupture. Branch scientists identified functional polymorphisms that increase the expression of MMP-9 and MMP-1 by amnion cells while genetic association studies indicated that the fetal carriage rate of these polymorphisms is associated with an increased risk of preterm PROM.

Congenital anomalies are the second leading cause of perinatal mortality in the United States, after premature birth. Advances in imaging techniques have permitted the use of ultrasound before birth to detect many anatomical defects. The branch is currently using three- and four-dimensional ultrasound to delineate normal fetal anatomy and improve the detection of congenital anomalies and estimation of fetal weight and growth. This year, the branch developed an approach for examining the fetus at risk for spina bifida and micrognathia and for using color power imaging to define normal vascular anatomy and identify congenital vascular disorders.

## PHYSICAL DISABILITIES BRANCH

**Steven J. Stanhope, Ph.D., Chief**

The Physical Disabilities Branch (PDB) was established in 2001 in part to accelerate the development of a highly collaborative, multi-disciplinary, technology development, and rehabilitation research network. To focus the network, the branch adopted a central programmatic research theme that stresses the decompartmentalization of rehabilitation research efforts. The Trans-Rehabilitation Research Domain (TRRD) model is based on the concept that our ability to understand entirely the process of both disablement and enablement is primarily predicated on our ability to link knowledge across the impairment, functional limitation, and physical disability rehabilitation research domains. During the past year, each of the three PDB sections contributed substantially toward achieving the branch's goals.

Scientists and engineers working within the Biomechanics and Biomedical Engineering Section develop and apply advanced technologies that create new directions for basic, translational, and clinical rehabilitation research in support of the TRRD. Their recent accomplishments include the development of a technique that extends existing movement analysis methodology by using a generalized coordinate-based mechanical linkage model that can provide direct estimates of the influence of a given joint moment or muscle force on the movement of all joints, body segments, and overall task performance. In addition, PDB staff and affiliates have demonstrated the feasibility of studying musculoskeletal kinematics with cine-Phase Contrast (PC) and fast-PC magnetic resonance imaging techniques.

The Human Movement Disorders Section is dedicated to conducting translational and clinical rehabilitation research. Its mission is to study and understand human function and the influence of rehabilitation interventions in the context of activities of daily living, with the goal of optimizing function for people with physical disabilities across the life span. Recent accomplishments include documenting the movement patterns and movement control strategies of patients with osteogenesis imperfecta, Parkinson's disease, progressive supranuclear palsy, juvenile dermatomyositis, and rheumatoid arthritis and identifying various lower extremity movement control strategies during gait that develop in response to various distribution patterns and severity of lower extremity muscle weakness.

The Oral Motor Function Section, directed by *Dr. Barbara Sonies*, strives to understand normative and adaptive oral motor functions that have an impact on clinical treatments and aid in the discovery of new strategies that may improve the lives of persons with swallowing, speech, and oral motor conditions. Recent accomplishments include documenting differences in the growth and maturation of the upper aerodigestive system between normal and at-risk fetuses; the identification of unique lingual perfusion and reperfusion patterns as a function of physiological task performance; the determination that normal individuals use 16 to 30 percent of their maximal voluntary isometric lingual-to-pallet pressure when swallowing; and documenting a unique pattern of cyclical "activation and partial deactivation" during rapid sequential swallowing.

## SECTION ON DNA REPLICATION, REPAIR AND MUTAGENESIS

**Roger Woodgate, Ph.D., Chief**

Scientists within the Section on DNA Replication, Repair and Mutagenesis are trying to understand the mechanisms by which mutations are introduced into damaged DNA. Recent studies have shown that many of the proteins long implicated in the mutagenic process are, in fact, low-fidelity DNA polymerases that can replicate damaged DNA. This so-called "Y-family" of DNA polymerases is found in bacteria, archaea, and eukaryotic cells, indicating that the mutagenic process is remarkably conserved throughout evolution. In addition to replicating damaged DNA, the polymerases exhibit low-fidelity synthesis on undamaged DNA and may be a major source of spontaneous mutations. Of particular interest is human DNA polymerase iota (polι), which was discovered by scientists within the section, and exhibits the unique property of inserting guanine rather than the canonical base adenine opposite template thymine. Interestingly, although polι is phylogenetically related to polη (which is best characterized for its ability to replicate past *cis-syn* thymine dimers both accurately and efficiently and is known to be defective in humans afflicted with the xeroderma pigmentosum variant phenotype), *in vitro* replication assays revealed that polι-dependent lesion bypass is remarkably inefficient and inaccurate. The exception was at a benzopyrene diol expoxide deoxyadenosine adduct, where polι efficiently inserted the correct base, dTMP. These *in vitro* studies suggest that one cellular function of polι might be to help protect humans from the mutagenic and carcinogenic affects of exposure to benzopyrene diol epoxides (which are normally found at high levels in cigarette smoke). *In vivo* studies with a fluorescent-tagged polι protein revealed that the enzyme is predominantly localized to the nucleus, where it associates with the cell's normal replication machinery. Following DNA damage, polι accumulates into discrete foci at sites of stalled replication forks. The pattern of foci formation was identical to that previously reported for the polη, suggesting that damage-induced polι- and polη-foci formation is tightly coordinated within the cell. Using a variety of techniques, scientists within the section discovered that polι and polη interact with each other physically and may, in fact, coexist in a larger holoenzyme complex where their lesion-bypassing activities can be coordinated in response to DNA

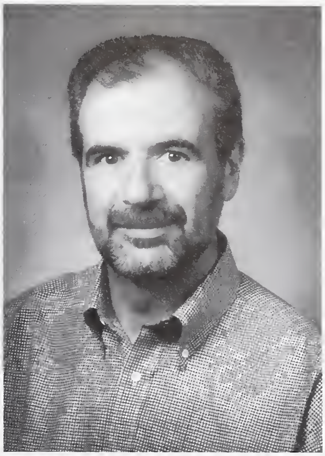
Another Y-family polymerase discovered and characterized by members of the group is the archaeal DNA polymerase, Dpo4, which shares biochemical properties with both its prokaryotic and eukaryotic orthologs. Previously, a collaborative study determined the crystal structure of Dpo4 in a ternary complex with undamaged DNA and an incoming nucleotide. In the past year, these structural studies have been extended to lesion-containing DNA, where they serve as a paradigm for understanding the molecular mechanisms of translesion replication promoted by all Y-family DNA polymerases.

The Institute continues to mount an ambitious and highly regarded Intramural Program with a continuing increase in the quality and quantity of its scientific productivity. The challenge for the future is to maintain this desirable trend and build on current directions of scientific inquiry.

Owen M. Rennert, M.D.
Scientific Director
National Institute of Child Health
and Human Development

# RESEARCH PROJECTS
# OF THE
# LABORATORIES
# AND
# BRANCHES

# PROTEIN SORTING IN THE ENDOSOMAL-LYSOSOMAL SYSTEM



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Steve Caplan, Ph.D., *Postdoctoral Fellow*
Bong-Yoon Kim, Ph.D., *Postdoctoral Fellow*
Jose Martina, Ph.D., *Postdoctoral Fellow*
Kengo Moriyama, M.D., *Postdoctoral Fellow*
Rosa Puertollano, Ph.D., *Postdoctoral Fellow*
Joost Drenth, M.D., *Guest Researcher*
Edna Chavez, *Technician*
Xiaolin Zhu, *Technician*

We investigate the molecular mechanisms that control the sorting of transmembrane proteins in the endosomal-lysosomal system. Sorting processes such as rapid internalization of receptors from the plasma membrane, targeting to lysosomes and related organelles, and delivery to the basolateral plasma membrane domain of polarized epithelial cells are all mediated by interactions between signals in the cytosolic domains of the transmembrane proteins and adaptor proteins associated with the cytosolic face of membranes. Two major types of sorting signal, referred to as tyrosine-based and dileucine-based, have been previously described. Work in our as well as in other laboratories has demonstrated that both types of signal are recognized with characteristic fine specificities by the adaptor protein (AP) complexes AP-1, AP-2, AP-3, and AP-4 or the GGA proteins GGA1, GGA2, and GGA3 (Fig. 1). Mutations in AP-3 are the cause of the pigmentation and bleeding disorder Hermansky-Pudlak syndrome type 2. Current work is aimed at elucidating the structure, regulation, and physiological roles of AP complexes and GGAs and at investigating the possibility that defects in these proteins underlie protein trafficking disorders.

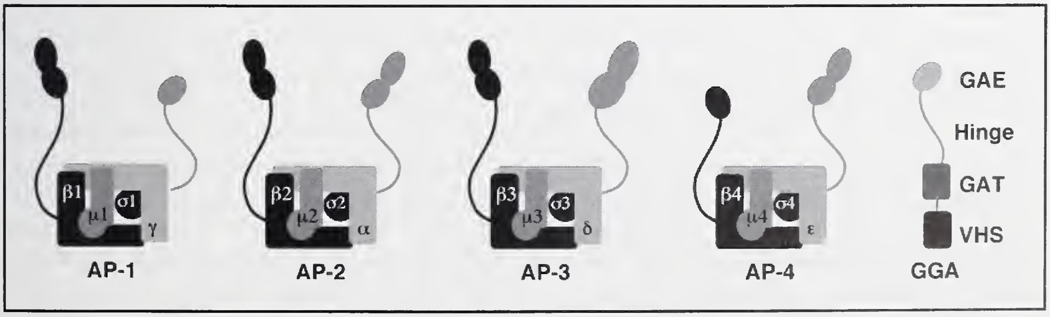

**FIGURE 1**
Structure of AP complexes and GGAs.

### Role of the GGAs in the Sorting of Lysosomal Hydrolases

*Puertollano, Bonifacino*

Over the past year, we continued our studies on the structure and function of the GGAs. The GGAs are modular proteins composed of four domains named the VHS, GAT, hinge, and GAE domains. Previous work in our laboratory demonstrated that the GGAs function as ARF-dependent adaptors for recruitment of clathrin to the TGN. We discovered that the VHS domain of the GGAs functions as a recognition module for dileucine-based sorting signals present in the cytosolic domains of the mannose 6-phosphate receptors (MPRs) that sort acidic hydrolases to lysosomes. In collaboration with James Hurley's group (NIDDK), we solved the crystal structure of a GGA VHS domain in complex with signals from the MPRs. The structure revealed the basis for the specificity of recognition of these signals. In addition, we demonstrated that casein kinase II–mediated phosphorylation of a serine residue within the MPR dileucine–based signal enhances interactions with the GGA VHS domain, thus explaining the regulation of this sorting process by phosphorylation. The studies have therefore solved the longstanding enigma of how the MPRs are able to sort acidic hydrolases to lysosomes. The knowledge gained from the biochemical and structural characterization of these interactions allowed us to predict a role for the GGAs in the sorting of other physiologically important proteins such as beta-secretase, the enzyme responsible for the generation of the beta-amyloid precursor associated with Alzheimer's disease.

### A Novel Type of Coated Vesicle Budding from the *Trans*-Golgi Network

*Puertollano, Bonifacino*

Sorting of MPRs and their hydrolase ligands at the *trans*-Golgi network (TGN) has long been thought to be mediated by small (60–100 nm diameter) clathrin-coated vesicles. We have conducted experiments using fluorescent imaging of live cells that suggest a role for a different type of clathrin-coated intermediate. Clathrin and GGA1, labeled with different spectral variants of the green fluorescent protein (GFP), colocalize to the TGN and to a population of vesicles budding from it. Strikingly, these vesicles are pleiomorphic and have average diameters of 360 to 380 nanometers. They move toward peripheral cytoplasm for distances of up to 10 micrometers with average speeds of about one micrometer per second. Independently of vesicle budding, the labeled clathrin and GGA1 cycle on and off membranes with half-times of 10 to 20 seconds. These observations suggest the existence of a novel type of clathrin-coated carrier that is both larger than conventional clathrin-coated vesicles and undergoes long-range translocation in the cytoplasm before losing its coat. The carriers are likely the intermediates that ferry acidic hydrolases from the TGN to the endosomal-lysosomal system.

### Interaction of TGN-Derived Carriers with Endosomes

*Mattera, Puertollano, Bonifacino*

The transfer of cargo from the clathrin- and GGA1-coated carriers to endosomes involves fusion or "kiss-and-run" interactions between these two types of organelle. We have identified a molecular interaction that may be responsible for the transfer. The GGAs have been found to bind via their GAE domains to the Rabaptin-5/Rabex-5 complex. The complex functions as a guanine nucleotide exchange factor for Rab5 and Rab4 and has been previously implicated in endosomal fusion reactions. The GAE domain binds to an FGPLV motif in the central region of Rabaptin-5.

### Biogenesis of Lysosome-Related Organelles

*Martina, Moriyama, Bonifacino*

We have previously shown that mutations in the gene encoding the beta3A subunit of AP-3 are the cause of Hermansky-Pudlak syndrome type 2, a disorder of lysosome-related organelles. Mutations in at least three other genes in humans and 14 genes in mice have been found to cause a similar disorder. Most of the HPS genes identified so far by positional cloning encode proteins of unknown function and no recognizable homology to other proteins. To gain insight into the nature of this machinery, we have undertaken a biochemical characterization of the novel HPS gene products. We have found that the protein products of the *pallid*, *muted*, and *cappuccino* genes are the components of a novel complex. The complex has two additional subunits of approximately 20kD and 15kD, which we are aiming to identify by affinity purification and mass spectrometry. Current studies are directed toward determining the intracellular localization and function of the complex. From these experiments, we hope to gain an understanding of the molecular mechanisms involved in the biogenesis of lysosome-related organelles and the pathogenesis of Hermansky-Pudlak syndrome.

### A Genomic Screen for Vacuolar Protein Sorting Genes in Yeast

*Bonangelino, Chavez, Bonifacino*

The yeast vacuole is the functional counterpart of mammalian lysosomes. Thus, yeast is an excellent model system for investigating the basic molecular mechanisms involved in lysosome biogenesis. To identify genes involved in vacuolar protein sorting, we conducted a genome-wide screen of 4,653 homozygous diploid gene deletion strains of *Saccharomyces cerevisiae* for missorting of the vacuolar hydrolase, carboxypeptidase Y (CPY). We identified 149 mutant strains that secreted strong to moderate levels of CPY. Of these, only 53 of the corresponding genes had been previously implicated in vacuolar protein sorting while the remaining 96 either had been identified in screens for other cellular processes or were known only as hypothetical open reading frames. Among the 96 are genes encoding (1) the four subunits of the AP-3 complex; (2) the Ras-like GTP-binding proteins, Arl1p and Arl3p; (3) actin-related proteins such as Arp5p and Arp6p; (4) the monensin and brefeldin A hypersensitivity proteins, Mon1p and Mon2p; and (5) 16 novel proteins designated Vps61p–Vps76p. Mutations in several of the novel proteins, including Vps61p, Vps64p, and Vps67p, resulted in actin cytoskeleton defects. The identification and phenotypic characterization of these novel mutants provide powerful new insights into the biogenesis of the yeast vacuole and mammalian lysosomes, most notably the involvement of the actin cytoskeleton in this process.

### A Tubular Intermediate in Protein Recycling to the Plasma Membrane

*Caplan, Bonifacino*

Pivotal to the function of many plasma membrane proteins is their ability to undergo endocytosis and recycling to the cell surface. We have uncovered a role for a protein named EHD1 in the recycling of plasma membrane proteins such as class I molecules of the major histocompatibility complex to the cell surface. EHD1 cooperates with the small GTP-binding protein ARF6 to induce the formation of tubules containing internalized proteins that extend from the center toward the periphery of the cells. The discovery of the role of EHD1 in this process opens the way for the identification of other components of the molecular machinery involved in recycling to the plasma membrane.

## SELECTED PUBLICATIONS

1. Boehm M, Bonifacino JS. Genetic analyses of adaptin function from yeast to mammals. Gene. 2002;286:175-186.
2. Bonangelino CJ, Chavez EM, Bonifacino JS. Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. Mol Biol Cell. 2002;13:2486-2501.
3. Bonifacino JS. Quality control of receptor-kinase signaling complexes. Dev Cell. 2002;2:1-2.
4. Caplan S, Naslavsky N, Hartnell L, Lodge R, Polishchuk RS, Donaldson JG, Bonifacino JS. A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. EMBO J. 2002;21:2557-2567.
5. Feng L, Novak EK, Hartnell L, Bonifacino JS, Collinson L, Swank RT. The Hermansky-Pudlak syndrome 1 (HPS1) and 2 (HPS2) genes independently contribute to the production and function of platelet dense granules, melanosomes and lysosomes. Blood. 2002;99:1651-1658.
6. Kato Y, Misra S, Puertollano R, Hurley JH, Bonifacino JS. Phosphoregulation of sorting signal-VHS domain interactions by a direct electrostatic mechanism. Nat Struct Biol. 2002;7:532-536.
7. Misra S, Puertollano R, Kato Y, Bonifacino JS, Hurley JH. Structural basis for acidic-cluster-dileucine sorting signal recognition by VHS domains. Nature. 2002;415:933-937.
8. Moriyama K, Bonifacino JS. Pallidin is a component of a multi-protein complex involved in the biogenesis of lysosome-related organelles. Traffic. 2002;3:666-677.
9. Puertollano R, Aguilar RC, Gorshkova I, Crouch RJ, Bonifacino JS. Sorting of mannose 6-phosphate receptors mediated by the GGAs. Science. 2001;292:1712-1716.
10. Puertollano R, Randazzo PA, Presley JF, Hartnell LM, Bonifacino JS. The GGAs promote ARF-dependent recruitment of clathrin to the TGN. Cell. 2001;105:93-102.

11. Wasiak S, Legendre-Guillemin V, Puertollano R, de Heuvel E, Bell AW, Bonifacino JS, McPherson PS. Enthoprotin, a novel clathrin-associated protein identified through subcellular proteomics. J Cell Biol. 2002;158:855-862.

## COLLABORATOR

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# REGULATION OF SECRETORY AND MEMBRANE PROTEIN BIOGENESIS

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Kelly Shaffer, *Predoctoral Fellow*
Jesse Yonkovich, *Predoctoral Fellow*
Ryen D. Fons, B.S., *Predoctoral Fellow*[a]
Gretchen A. Reinhart, B.S., *Predoctoral Fellow*[b]
Devarati Mitra, *Student*[c]
Samantha L. Schwartz, *Student*[c]

We study the mechanisms regulating the synthesis, translocation, and maturation of secretory and membrane proteins at the mammalian endoplasmic reticulum (ER). A complex macromolecular assembly at the ER, termed the translocon, serves as a protein-conducting channel where substrates enter the secretory pathway. The translocon participates in diverse cellular activities that range from the import of secretory proteins, to topogenesis and assembly of complex multi-spanning membrane proteins, to the export of misfolded substrates from the ER to the cytosol for degradation. The mechanisms that allow the shared translocon to accommodate such an extensive range of substrates efficiently yet accurately remain largely unknown. The principal goal of ongoing studies is to define the molecular mechanisms and components of the translocon that recognize the information in the primary sequence of its substrates to mediate their proper vectorial transport, asymmetric topogenesis, and membrane integration. By delineating the steps during the biosynthesis of normal versus disease-associated variants of secretory and membrane proteins, we are formulating and testing *in vivo* hypotheses regarding the molecular basis of particular diseases of the early secretory pathway.

## Molecular Mechanism of Prion Protein Topogenesis

*Kim*

The prion protein (PrP), a brain glycoprotein involved in various neurodegenerative diseases, has proven to be a particularly instructive example of complex and highly regulated translocation. In addition to its notoriety as the putative "protein-only" infectious agent in prion diseases, the biogenesis of PrP at the ER is unusual in that an initially homogeneous cohort of nascent PrP chains gives rise to three distinct topologic forms: a fully translocated form (termed $^{sec}$PrP) and two transmembrane forms that span the membrane in opposite orientations ($^{Ntm}$PrP and $^{Ctm}$PrP). *In vivo* studies have revealed that even a slight overrepresentation of the $^{Ctm}$PrP topologic form results in the development of neurodegenerative disease both in mouse-model systems and in the spontaneous forms of human disease. Our current efforts focus on dissecting the mechanisms that direct an initially homogeneous cohort of nascent PrP polypeptides into multiple topologic forms. We recently discovered that segregation of nascent PrP into different topologic forms depends critically on the precise timing of signal sequence–mediated initiation of N-terminus translocation. Consequently, the initiation step could be experimentally tuned to modify PrP topogenesis, including complete reversal of the elevated $^{Ctm}$PrP caused by disease-associated mutations in the transmembrane domain. We are now initiating studies in transgenic mice to determine whether $^{Ctm}$PrP-mediated neurodegeneration can be averted by modulating this newly discovered step during PrP biogenesis.

Parallel biochemical studies employing the solubilization, fractionation, and reconstitution of ER membrane proteins have demonstrated that regulatory *trans*-acting factors are absolutely required for PrP to be synthesized in the proper ratio of its topologic forms. We have now purified two such factors and identified them as the translocon-associated protein complex (TRAP) and protein disulfide isomerase (PDI). Analysis of PrP translocation intermediates suggests that TRAP and PDI act sequentially to facilitate translocation of PrP's N-terminus into the ER lumen, the decisive event in determining PrP topology. Ongoing studies are investigating the role of these newly discovered factors in the biogenesis of other substrates (see below) and their potential role in the pathogenesis of PrP-associated neurodegeneration.

### Regulation of Protein Biogenesis by Signal Sequences

*Kim, Shaffer, Mitra*

Our discovery that PrP topology is regulated by the N-terminal signal sequence has established a new function for this domain that is independent of the domain's well-studied role in protein targeting. Using signal sequence mutants that uncouple targeting and post-targeting functions, we demonstrated that the post-targeting function lies in gating the translocation channel to provide the nascent polypeptide with access to the ER lumen. We subsequently developed and used a novel assay for signal-mediated translocon gating to demonstrate that signal sequences display a remarkable degree of variation in initiating nascent chain access to the lumenal environment. Such substrate-specific properties of signals were found to be evolutionarily conserved, functionally matched to their respective mature domains, and important for the proper biogenesis of some proteins. A recent analysis of several naturally occurring disease-associated mutants in signal sequences have revealed that many mutants are altered in their gating function, and not in the targeting function, as previously assumed. Thus, we have discovered that the long-observed sequence variations of signals do not simply represent functional degeneracy but instead encode differences in translocon gating that are critical to the proper biogenesis of its attached substrate. We are currently investigating whether the substrate-specific properties of signal sequences have been exploited by the cell to regulate the subcellular localization of certain proteins, such as calreticulin, that have been shown to be present in multiple compartments.

### A Function for TRAP in Substrate-Specific Protein Translocation

*Rane, Yonkovich, Hegde, Fons*

The search for factors involved in PrP topogenesis led to our purification of the TRAP complex, a set of four proteins with a previously unknown function. Our recent studies established that TRAP is required for the translocation of some, but not other, substrates. The substrate-specificity proved to be encoded in the signal sequence. Remarkably, TRAP specifically aids vectorial transport of substrates whose signal sequences, after mediating targeting to the ER, are delayed in their gating of the translocon. Thus, it appears that TRAP is a key component of the translocation machinery that aids in decoding the substrate-specific information in signal sequences. Our current studies of TRAP function focus on understanding the molecular mechanism by which it facilitates signal recognition, substrate translocation, and protein topogenesis. In addition, we are investigating the consequence *in vivo* of RNAi-mediated suppression of TRAP expression in cultured cells.

### The Role of ER Chaperones in PrP Topogenesis

*Kim, Hegde, Fons*

Cross-linking analysis of PrP translocation intermediates has identified several proteins that are presumably involved in some aspect of its biogenesis. By comparing cross-linking patterns for different topology-altering mutants of PrP, we were able to identify several lumenal chaperones, including PDI, as proteins that selectively cross-link to translocation intermediates of $^{sec}$PrP. Biochemical extraction of the lumenal contents from ER-derived microsomes resulted in membranes that preferentially generate the transmembrane forms of PrP, suggesting that lumenal chaperones are required for proper PrP translocation. We have recently discovered that, under conditions of ER stress in cultured cells, when lumenal chaperones are functionally depleted by unfolded substrates, newly synthesized PrP appears to be improperly translocated. Importantly, the transport of certain other substrates is not impaired, arguing against a global defect in translocation. Given that dysregulation of PrP topogenesis can lead to neurodegeneration, our findings suggest a link between cellular stress, PrP topology, and disease pathogenesis. We are currently investigating whether titration of ER chaperones during prion disease may play a role in neurodegeneration through the inappropriate topogenesis of PrP.

### Visualization of Translocon Organization in Cells

*Hegde, Reinhart, Schwartz; in collaboration with Snapp, Lippincott-Schwartz*

A qualitatively different facet of protein biogenesis is the question of *where* within the ER various events occur. The translocon participates in diverse cellular activities that range from the import of secretory proteins, to topogenesis and assembly of complex multi-spanning membrane proteins, to the export of misfolded substrates from the ER to the cytosol for degradation. We wish to determine whether all these translocon-associated activities are homogeneously distributed throughout the ER or whether they are organized and regulated in the spatial and temporal dimensions to meet the changing needs of the cell. There is presently little or no insight into this question largely because the current approaches to understanding protein translocation use biochemical systems in which spatial relationships are lost. In collaboration with the laboratory of Jennifer Lippincott-Schwartz, we are using biophysical techniques such as fluorescence resonance energy transfer (FRET) to probe *in situ* the molecular organization of the components of the translocation machinery. In initial studies, we used analysis of FRET between subunits of the Sec61p complex, a principal component of the ER protein translocon, to monitor directly the assembly state of the translocon in cells. Our studies have revealed that, while the translocon can be assembled from its components in response to ligands for protein translocation in biochemical systems, it does not disassemble and reassemble between successive rounds of transport *in vivo*. Instead, an actively engaged translocon is distinguished from a quiescent translocon by conformational changes, which can be directly detected by differences in FRET. By correlating the formation of particular protein complexes with biochemical activities, we endeavor to visualize directly the functional segregation and organization of the ER and to monitor potential changes in it during cellular metabolism, development, or disease pathogenesis.

## SELECTED PUBLICATIONS

1. Hegde RS. Targeting and beyond. New roles for old signal sequences. Mol Cell. 2002;10:697-698.
2. Kim SJ, Hegde RS. Cotranslational partitioning of nascent prion protein into multiple populations at the translocation channel. Mol Biol Cell. 2002;13:3775-3786.
3. Kim SJ, Mitra D, Salerno JS, Hegde RS. Signal sequences control gating of the protein translocation channel in a substrate-specific manner. Dev Cell. 2002;2:207-217.
4. Kim SJ, Rahbar R, Hegde RS. Combinatorial control of prion protein biogenesis by the signal sequence and transmembrane domain. J Biol Chem. 2001;276:26132-26140.
5. Kim SS, Zhang R, Braunstein SE, Joachimiak A, Cvekl A, Hegde RS. Structure of the retinal determination protein dachshund reveals a DNA binding motif. Structure (Camb). 2002;10:787-795.
6. Lingappa VR, Rutkowski DT, Hegde RS, Andersen OS. Conformational control through translocational regulation: a new view of secretory and membrane protein folding. Bioessays. 2002;24:741-748.
7. Rutkowski DT, Lingappa VR, Hegde RS. Substrate-specific regulation of the ribosome-translocon junction by N-terminal signal sequences. Proc Natl Acad Sci USA. 2001;98:7823-7828.

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[a]Left NICHD August, 2002
[b]Left NICHD July, 2002
[c]Left NICHD September, 2002

# CELL CYCLE REGULATION IN OVARIAN CYSTS

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Takako Iida, Ph.D., *Postdoctoral Fellow*
Isamu Sugimura, Ph.D., *Postdoctoral Fellow*

We use *Drosophila* oogenesis as a model to explore the developmental regulation of the cell cycle. The long-term goal of the laboratory is to understand how the cell cycle program of the *Drosophila* ovarian cyst is coordinated with the developmental events of oogenesis. The *Drosophila* oocyte develops within the context of a 16-cell germline cyst. Individual cells within the cyst are referred to as cystocytes and are connected by actin-rich ring canals. The cyst is produced through a series of four synchronous mitotic divisions during which cytokinesis is incomplete. While all 16 cystocytes enter premeiotic S phase, only a single cell remains in the meiotic cycle and becomes the oocyte. The other 15 cells enter the endocycle and develop as highly polyploid nurse cells. Currently, we are working to understand how cells within ovarian cyst enter and maintain either the meiotic cycle or the endocycle. In addition, we are examining how such cell cycle choice influences the nurse cell/oocyte fate decision.

## *missing oocyte*, a New Gene Required for the Maintenance of the Meiotic Cycle

*Iida*

To identify the pathways that direct entry into and maintenance of the meiotic cycle in the single pro-oocyte, we screened for mutants in which all 16 cells enter the endocycle and develop as nurse cells. From the screen, we identified a new gene, *missing oocyte* (*mio*), that is required for the maintenance of the meiotic cycle. In *mio* mutants, the oocyte enters the meiotic cycle, forms mature synaptonemal complexes, and progresses to pachytene. However, the meiotic state is not maintained. *mio* oocytes eventually abandon the meiotic cycle, enter the endocycle, and develop as nurse cells.

We have characterized the *mio* gene at the molecular level. *mio* is predicted to encode a protein of 867 amino acids; the protein contains four WD40 repeats and is conserved from yeast to humans. We have determined that the Mio protein is present at high levels in the oocyte nucleus soon after it enters prophase of meiosis I. Double labeling with anti-antibodies and an antibody against the synaptonemal complex protein C(3)G indicates that Mio specifically localizes to the nucleus of the oocyte soon after the completion of premeiotic S phase. As a result, for the *Drosophila* oocyte, Mio stands apart as one of the earliest nuclear markers that is not a known component of the synaptonemal complex.

Genetic interaction studies indicate that *mio* functions early in meiosis, before the onset of pachytene. In *egalitarian* (*egl*) mutants, all 16-cyst cells enter the meiotic cycle and progress to pachytene as assayed by the presence of mature synaptonemal complexes. However, the meiotic state cannot be maintained, and eventually all the cells exit meiosis and enter the endocycle. Germline cysts from *mio*, *egl*$^{null}$ double mutants have a significantly stronger phenotype. The *mio, egl*$^{null}$ double mutants never form mature synaptonemal complexes and do not progress past zygotene. The data indicate that *mio* influences the oocyte cell cycle in early prophase of meiosis I; however, precisely when *mio* is required to maintain the meiotic cycle remains to be determined.

In further support of a role for *mio* in early meiosis, mutations in *mei-W68* and *mei-P22* suppress the *mio* 16-nurse–cell phenotype. *mei-W68* and *mei-P22* are required for the production of double-stranded breaks during meiosis. In addition, *mio* is partially suppressed by mutations in *mei41*. *mei41* is required for the prophase I meiotic checkpoint arrest. The data suggest that *mio* may interact with pathways that influence DNA metabolism or the meiotic checkpoint. Further studies of *mio* may help elucidate the poorly characterized pathways that control meiotic progression and the maintenance of oocyte identity.

### Two Novel Functions for the p27$^{KIP1}$-Like CDK Inhibitor Dacapo in Ovarian Cysts

*Hong, Iida, Sugimura*

*dacapo* is a vital gene that encodes a p21$^{CIP}$/p27$^{KIP1}$/p57$^{KIP2}$-like cyclin-dependent kinase inhibitor (cki), which specifically inhibits the activity of CycE/Cdk2 complexes. CycE/Cdk2 activity is required for S phase in *Drosophila*. Other researchers have been shown that, throughout much of the growth phase of *Drosophila* oogenesis, the levels of the cki Dacapo oscillate in the 15-polyploid nurse cells but remain persistently high in the single oocyte. We have demonstrated that both modes of Dacapo regulation are functionally important. In the oocyte, the prophase I arrest is lost or not properly established in germline cysts that lack Dacapo. This is the first demonstration of a cip/kip family member functioning in a normal meiotic cycle. In addition, our data indicate that Dacapo is part of the biochemical oscillator that drives the nurse-cell endocycle. Specifically, we find that, in polyploid nurse cells, the oscillations of Dacapo facilitate the relicensing of DNA replication origins during endoreplication by inhibiting CycE/Cdk2 activity at the end of each endocycle S phase. Our data are consistent with recently proposed models that suggest the periodic expression of members of the cip/kip family of Cdk inhibitors' direct entry into the Gap phase during endoreplicative cycles. We propose that it is through the differential regulation of the cki Dacapo that two dramatically different cell cycles, the meiotic cycle and the endocycle, are independently maintained within the common cytoplasm of the ovarian cyst.

### The *Drosophila* Fusome Contains a Highly Interconnected Endoplasmic Reticulum

*Iida; in collaboration with Lippincott-Schwartz*

We have entered into collaboration with the laboratory of Jennifer Lippincott-Schwartz to use live imaging of proteins with green fluorescent protein (GFP) to study the structure and function of the *Drosophila* fusome. The fusome is a membranous organelle comprised of microtubules and an array of membrane-associated cytoskeletal proteins; it grows along the remnants of the mitotic spindle after each cyst division. In the absence of the fusome, which physically connects all the cells in the cyst, mitotic synchrony between cystocytes is lost, the number of cystocyte divisions is reduced, and oocyte differentiation does not occur. However, the molecular mechanisms by which the fusome influences the cystocyte cell cycle and oocyte differentiation are poorly understood.

To understand more fully the origin and function of the membrane component of the fusome, we generated transgenic lines carrying a lysozyme-GFP-KDEL (lys-GFP-KDEL) chimera. In mammalian cells, the chimera is retained in the endoplasmic reticulum (ER) lumen by the COOH-terminal KDEL motif, which, in conjunction with the KDEL receptor, targets soluble proteins to the ER lumen. The targeting system is highly conserved. The *Drosophila* ortholog of the well-studied ER lumenal protein BiP contains the KDEL targeting sequence. Consistent with the expected location of the ER, our observation of live germaria indicates that the lys-GFP-KDEL protein targets to a series of whip-like tubules in the cytoplasm of germline cells. In addition to localization in cytoplasmic structures, lys-GFP-KDEL targets to thick branching structures that resemble fusomes. We confirmed that the lys-GFP-KDEL chimera targets to fusomal membranes by fixing and staining the lys-GFP-KDEL–expressing ovaries with the anti-fusome marker Hts. The targeting of the lys-GFP-KDEL transgene to the fusome strongly suggests that the fusomal membranes arise from the ER.

Electron micrographs suggest that fusomal membranes are discontinuous fragments. Reports of fragmented ER structures in other organisms are extremely rare, and live cell imaging studies have often revealed these fragmented ER structures to be fixation artifacts. To probe fusome membrane continuity, we performed fluorescence recovery after photobleaching (FRAP) of the GFP-chimeras in fusomes. Both visually and quantitatively, the GFP-chimera can be observed rapidly recovering into the photobleached region of the fusome. The fluorescence recovery occurs on both sides of the bleached region, indicating that recovery is not directional. This rapid bidirectional recovery is inconsistent with the fact that the fusome is composed of discrete membranous compartments.

Finally, we performed fluorescence loss in photobleaching (FLIP) to assay the continuity of fusome and cytoplasmic ER membranes throughout the cyst. FLIP involves repeatedly photobleaching a small region of interest. If proteins within the photobleached region are completely mobile and the entire structure is continuous with the photobleached region, then the structure's fluorescence will be depleted over time. When a small region of cytoplasmic ER from a single cystocyte was repeatedly photobleached, the ER in the entire cyst, including the fusome, rapidly lost fluorescence, whereas adjacent cysts were unaffected.

Our work has led to three important conclusions: the fusomal membranes are derived from the ER; the fusome is continuous with the cytoplasmic ER; and all the cells within a single ovarian cyst share a common ER. We predict that the interconnectivity of the cyst ER, through the fusome, may be important to the synchronization of the mitotic cyst divisions as well as to other signalling events within the cyst.

### *twin*, Putative Ortholog of the Yeast CCR4 Gene

*Hong; in collaboration with Lehmann*

Mutations in the *twin* gene cause an array of phenotypes that suggest that the cell cycle program of the ovarian cyst has been altered. First, *twin* egg chambers frequently contain either too few or two many cells, indicating that the cyst has undergone an inappropriate number of mitotic divisions. Second, approximately five percent of *twin* 16-cell cysts contain two oocytes. Finally, the nurse cells in *twin* mutants frequently exhibit abnormal chromatin structure. In collaboration with Ruth Lehmann's laboratory, we have determined that *twin* is the putative ortholog of the *S. cerivisiae* CCR4 gene. In yeast, CCR4 is the catalytic subunit of the major cytoplasmic mRNA deadenylase. Deadenylation influences both mRNA function and stability. In addition, CCR4 has been implicated in transcriptional initiation and elongation. The catalytic domain, which contains 3'-5'poly(A)RNA and ssDNA exonuclease activity, is highly conserved between CCR4 and *twin*. We are currently investigating whether the *twin* phenotype is dominantly modified by mutations in known cell cycle genes and by components of the transcriptional machinery that genetically interact with CCR4 in yeast.

## PUBLICATION

Lilly MA, de Cuevas M, Spradling AC. Cyclin A associates with the fusome during germline cyst formation in the *Drosophila* ovary. Dev Biol. 2000; 218:53-63.

## COLLABORATORS

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# REGULATION OF MEMBRANE DYNAMICS AND PROTEIN TRANSPORT BY THE Sec7 DOMAIN GUANINE NUCLEOTIDE EXCHANGE FACTORS

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We study the Sec7 domain guanine nucleotide exchange factors (GEFs) for the Arf family of small GTPases. We are interested in the roles of these proteins in membrane dynamics and protein trafficking. The Arfs and the Arf GEFs are important regulators of both organelle structure and protein transport throughout the cell. We are focusing our attention on the large Golgi-localized Arf GEFs involved in transport through the secretory pathway in both budding yeast and mammalian cells. A central question in cell biology is how the elaborate and dynamic structures of membrane systems are maintained in the face of constant trafficking into and out of each organelle. Particularly important questions include the way organelle structure is generated and maintained and how structure is correlated with the underlying molecular events of protein sorting and membrane remodeling. An important step in resolving these issues is to define the roles of the Arf GEFs at the molecular level through the identification of interacting partners, elucidation of membrane localization mechanisms, and analysis of Arf GEF mutants *in vivo*.

## Identification of Interacting Partners of the Golgi-Localized Arf GEFs in Yeast and Mammalian Cells

*Smirnova, Park, Niu, Phillips*

The Arf GEFs of the Golgi-localized Gea/GBF and Sec7/BIG subfamilies are large multidomain proteins. A major goal of our laboratory is to understand the functions of the different domains of the Gea/GBF and Sec7/BIG Arf GEFs. Given that the GEFs are soluble proteins that must be targeted to membranes, identification of their membrane-targeting signals and partners is an important step in understanding their function. Within each subfamily, regions upstream and downstream of the Sec7 catalytic domain are conserved from yeast to humans. However, the functions of these homology regions remain unknown. We are carrying out two-hybrid screens with the N-terminal and C-terminal regions of the Arf GEFs of the Gea/GBF and Sec7/BIG subfamilies. We carried out a two-hybrid screen using the Sec7 domain of the mammalian homolog of Sec7p, BIG2, and found a number of partners, two of which are human PI4P 5 kinase β and a putative lipase. PI4P 5 kinase β is an extremely interesting potential partner in that it has been identified as an effector of Arf. We carried out a reverse two-hybrid screen to determine the region of the BIG2 Sec7 domain that interacts with PI4P 5 kinase. Interestingly, we found that residues in the C-terminal region of the Sec7 domain abolish interaction with PI4P 5 kinase. We have narrowed down the interaction domain of PI4P 5 kinase to a 50-amino acid region in the C-terminal portion of the catalytic domain just upstream of the activation loop. The activation loop contains the substrate binding site and is both necessary and sufficient to determine intracellelular localization of the kinase. We are also mapping the region of Arf interaction in PI4P 5 kinase. Interestingly, it appears to be located in a different domain of the protein than the BIG2 Sec7 domain–interacting region. It is striking that we have identified three lipid-modifying enzymes as binding partners of different Sec7 domains. It appears that all three proteins bind to the C-terminal region of the Sec7 domain. We know that the region is adjacent to a PH domain in the ARNO subfamily of Arf GEFs, which mediates membrane localization through specific lipid interaction. Using the Gea2p N-terminal region, we have identified Mss4p as an interacting partner. Mss4p is the sole PI4P 5 kinase in yeast. It is intriguing that we identified a human homolog of Mss4p in our two-hybrid screen with the BIG2 Sec7 domain.

We have identified a five-span trans-membrane domain protein, Msg1p, as a potential membrane receptor for the Gea1p and Gea2p proteins. Msg1p was originally identified as a multicopy suppressor of the temperature-sensitive growth defect of the *gea1-6* mutant, and it suppresses the cold-sensitive growth defect of *arf1Δ* when overexpressed. The *gea1-6* mutant has two point mutations downstream of the Sec7 domain in the C-terminal region of the protein. Msg1p is highly conserved from yeast to humans, and both the yeast and human proteins are localized to the Golgi. In yeast, Msg1p co-localizes with Gea2p and with early Golgi markers. Msg1p interacts with the C-terminal region of both Gea1p and Gea2p by two-hybrid analysis, but not with the C-terminus of *gea1-6*. The results support the idea that Msg1p is a Golgi-localized membrane receptor for the Gea proteins.

**Morphology of the Yeast Secretory Pathway**

*Park, Rainey; in collaboration with Hartnell, Rambourg*

The Arf GEFs are important regulators of organelle structure and protein trafficking in both yeast and mammalian cells. Yeast organelles are seemingly differ dramatically in structure and organization from those of mammalian cells, yet most of the proteins involved in organelle structure and trafficking, including the Arf GEFs, are highly conserved. Current work is aimed at determining Golgi structure in yeast by using both live imaging and electron microscopy as a first step in identifying the structural features common to yeast and mammalian organelles. Use of both the yeast and mammalian systems will allow us to determine which aspects of Arf GEF function are fundamental to all eukaryotic organisms and which are unique to their specific system.

The molecular details of transport through the secretory pathway in the yeast *Saccharomyces cerevisiae* have been elegantly worked out. However, technical limitations have prevented the clear specification of the spatial organization of these molecular events. Organelles of the secretory pathway, in particular the Golgi apparatus, are difficult to image at both the light and electron microscopy levels. The difficulty results from the small size of yeast cells and the structure of the yeast Golgi apparatus, which is composed of approximately 30 dispersed elements scattered throughout the cytoplasm. The size of a yeast cell is only an order of magnitude above the wave length of visible light, thereby limiting the resolution of intracellular structures. It is therefore necessary for any structural analysis in yeast to include electron microscopy. We are collaborating with Lisa Hartnell to perform immuno-electron microscopy of the yeast secretory pathway. Previous work in collaboration with Alain Rambourg indicated that the large, ring-like structures that accumulate in the *gea1-4* mutant are made up of tubular networks. Tubular networks of a similar type are also found in wild-type cells, although the structures are generally smaller. We were able to visualize the Och1-HA–containing structures in the *gea1-4* mutant and found that they were very similar to those visualized by Rambourg's reduced osmium technique. We were also able to obtain a good level of signal for the wild-type strain despite the fact that the Och1-HA–containing structures are smaller. We are currently carrying out co-localization experiments with early and late Golgi markers to determine whether they are present on completely separate Golgi elements or whether they can be present in different areas of the same continuous structure. We are also carrying out live imaging of secretory pathway proteins by using GFP-tagged markers.

## SELECTED PUBLICATIONS

1. Chantalat S, Courbeyrette R, Senic-Matuglia F, Jackson CL, Goud B, Peyroche A. A novel Golgi-localized integral membrane partner of the ARF GEFs Gea1p and Gea2p. J Cell Biol. 2002;in press.
2. Jackson CL. The Sec7 family of ARF guanine nucleotide exchange factors, chapter IV. In: Kahn RA, ed. The ARF book. Kluwer Academic Publishers 2002;in press.
3. Mayer G, Blind M, Nagel W, Böhm T, Knorr T, Jackson CL, Kolanus W, Famulok M. Controlling small guanine-nucleotide-exchange factor function through cytoplasmic RNA intramers. Proc Natl Acad Sci USA. 2001;98:4961-4965.
4. Peyroche A, Courbeyrette R, Rambourg A, Jackson CL. The ARF exchange factors Gea1p and Gea2p regulate Golgi structure and function in yeast. J Cell Sci. 2001;114:2241-2253.
5. Peyroche A, Jackson CL. Functional analysis of ADP-ribosylation factor (ARF) guanine nucleotide exchange factors Gea1p and Gea2p in yeast. Methods Enzymol. 2001;329:290-300.
6. Rambourg A, Jackson CL, Clermont Y. Three dimensional configuration of the secretory pathway and segregation of secretion granules in the yeast Saccharomyces cerevisiae. J Cell Sci. 2001;114:2231-2239.

## COLLABORATORS

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# REGULATORY RNAS AND MICROBIAL RESPONSE TO OXIDATIVE STRESS

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Our two main interests are the characterization of the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress and transcriptional regulators whose activities are modified by oxidation and reduction and the identification and characterization of all *E. coli* noncoding RNAs.

**Microbial Responses to Oxidative Stress**

*Storz, Mukhopadhyay, Outten, Woods, Carmel-Harel, Andrade*

One focus of the group is to study how organisms sense environmental signals and transduce the signals into changes in gene expression and cell physiology. Specifically, we are examining the *E. coli* and *S. cerevisiae* responses to oxidative stress. Reactive oxygen species can lead to the damage of almost all cell components (DNA, lipid membranes, and proteins) and have been implicated as causative agents in several degenerative diseases. Most organisms have an adaptive response to defend against oxidants. For example, treatment of both bacterial and yeast cells with low doses of hydrogen peroxide results in the induction of a distinct group of proteins, the decreased expression of other proteins, and resistance to killing by subsequent higher doses of hydrogen peroxide.

In bacterial cells, the key regulator of the inducible defenses against hydrogen peroxide is the OxyR transcription factor. We discovered that OxyR is both the sensor and transducer of the oxidative stress signal: the oxidized but not the reduced form of the purified regulator can activate transcription *in vitro*. OxyR is activated by the formation of an intramolecular disulfide bond between C199 and C208 and is deactivated by enzymatic reduction by glutaredoxin 1 together with glutathione. Structural studies showed that formation of the C199–C208 disulfide bond leads to a large conformational change. Computational and microarray experiments allowed us to identify many of the genes regulated by OxyR.

We now are examining the chemical basis of OxyR sensitivity to hydrogen peroxide and the roles of all of OxyR target genes. Compared with the bacterial response to hydrogen peroxide, less is known about the cellular mechanisms used by higher cells to sense and protect against oxidative damage. To initiate studies of the oxidative stress response in eukaryotes, we constructed isogenic *S. cerevisiae* strains carrying mutations in known signal transduction pathways and compared the oxidant sensitivities and whole genome expression patterns of these mutants. The studies confirmed that the Yap1 transcription factor is critical for resistance to hydrogen peroxide. We have purified the Yap1 protein and have begun biochemical experiments to characterize this redox-sensitive transcription factor. To identify new components of the eukaryotic response to oxidative stress, we also have been carrying out genetic screens for yeast mutants with altered sensitivities to hydrogen peroxide.

### Regulatory RNAs

*Storz, Zhang, Kawano*

A second focus of the group is to identify untranslated, regulatory RNAs and to elucidate their functions. These noncoding RNAs have been shown to have roles in transcriptional regulation, chromosome replication, RNA processing and modification, mRNA stability and translation, and even protein degradation and translocation. Until recently, however, most noncoding RNAs were discovered fortuitously. Noncoding RNA genes are usually poor targets in genetic screens and have been difficult to detect by direct sequence inspection.

We have been characterizing two previously identified *E. coli* regulatory RNAs, OxyS and 6S RNA, as well as carrying out screens for additional noncoding RNAs. The OxyS RNA, whose expression is induced by OxyR in response to oxidative stress, acts as both a global regulator that activates and represses the expression of multiple genes and an antimutator that protects cells against DNA damage. Studies of the *fhlA* and *rpoS* targets showed that the OxyS RNA represses translation of these genes. We recently found that OxyS RNA action depends on the Sm-like Hfq protein and that Hfq functions as a chaperone to facilitate OxyS RNA basepairing with its target mRNAs. We also discovered that another abundant noncoding RNA, 6S RNA, binds and modifies RNA polymerase. In addition, we used comparative genomics and microarrays to identify other noncoding RNAs in *E. coli*. These approaches led to the identification of 17 new regulatory RNAs whose functions we are now characterizing.

## SELECTED PUBLICATIONS

1. Gottesman S, Storz G, Rosenow C, Majdalani N, Repoila F, Wassarman KM. Small RNA regulators of translation: mechanisms of action and approaches for identifying new small RNAs. Cold Spring Harb Symp Quant Biol. 2002;66:353-362.
2. Storz G. An expanding universe of non-coding RNAs. Science. 2002;296:1260-1263.
3. Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G. The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. Mol Cell. 2002;9:11-22.

## COLLABORATORS

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# DYNAMICS OF SECRETORY MEMBRANE TRAFFICKING, SORTING, AND COMPARTMENTALIZATION

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We investigate the global principles underlying secretory membrane trafficking, sorting, and compartmentalization within eukaryotic cells. We are using live cell imaging of green fluorescent protein (GFP) fusion proteins in combination with photobleaching and photoactivation techniques to investigate the subcellular localization, mobility, transport routes, and binding interactions of a variety of proteins with important roles in the organization and regulation of membrane traffic and compartmentalization. To test mechanistic hypotheses related to protein and organelle dynamics, we use quantitative measurements of protein characteristics in kinetic modeling and simulation experiments. Among the topics currently under study are growth and maintenance of endoplasmic reticulum (ER) and Golgi morphology in mammalian cells and in developing *Drosophila* embryos; the mechanism(s) of secretory protein transport into and out of the Golgi apparatus; membrane binding/dissociation kinetics of trafficking machinery and its regulation; the generation and maintenance of cell polarity; and organelle breakdown and reassembly during mitosis. We have also recently developed a photoactivatable GFP whose mechanism of photoactivation is currently under investigation.

## Development of a Photoactivatable GFP

*Patterson*

Photoactivation is the rapid conversion of photoactivatable molecules to a fluorescent state by intense irradiation; it is a useful method for marking and monitoring selected molecules within cells. Previous efforts to develop a photoactivatable protein capable of high optical contrast when photoactivated under physiological conditions have had limited success. By mutagenizing wild-type GFP (wtGFP), we have developed a variant of GFP that allows selective marking of proteins through photoactivation. The wtGFP normally exists as a mixed population of neutral phenols and anionic phenolates producing major 397 nm and minor 475 nm absorbance peaks, respectively. Upon intense illumination of the protein with ultraviolet or ~400 nm light, the chromophore population undergoes photoconversion and shifts predominantly to the anionic form, giving rise to an increase in minor peak absorbance. The result is an approximately three-fold increase in fluorescence upon excitation at 488 nm. The photoactivatable variant of wt GFP that we developed (called PA-GFP for "photo" and "activatable") contains a histidine substitution at the T203 position of GFP, which results in a negligible minor absorbance peak. Photoconversion with irradiation of about 400 nm produces a large increase in absorbance at the minor peak and thus a more noticeable optical contrast under 488 nm excitation.

Upon photoactivation of living cells, PA-GFP exhibited an optical enhancement of nearly two orders of magnitude, making it suitable for marking specific protein or cell populations. Furthermore, the speed with which an optical signal was obtained and the absence of signal from newly synthesized proteins indicated that PA-GFP photoactivation was a labeling method preferable to photobleaching for studying the temporal and spatial dynamics of proteins. We used the photoactivatable GFP as both a free protein to measure protein diffusion across the nuclear envelope and as a chimera with a lysosomal membrane protein to demonstrate rapid interlysosomal membrane exchange. Our results suggest that the photoactivatable variant of GFP, PA-GFP, has the potential for addressing many fundamental questions in cell and developmental biology.

### Dissection of COPI Dynamics *in Vivo*

*Presley, Liu*

Cytosolic coat proteins that bind reversibly to membranes have a central role in membrane transport within the secretory pathway. One well-studied example is COPI or coatomer, a heptameric protein complex that is recruited to membranes by the GTP-binding protein Arf1. Assembly into an electron-dense coat then helps in budding off membrane to be transported between the ER and Golgi. To study COPI dynamics *in vivo*, we fused variants of GFP to the carboxy terminus of the εCOPI subunit of coatomer for expression in ldlF cells, which contain a mutated εCOP that is degraded at 40°C, causing coatomer inactivity and growth inhibition. The cells grew indefinitely at 40°C, indicating that εCOP-GFP could substitute functionally for endogenous εCOPI in coatomer complexes in these cells.

We studied the identity and behavior of coatomer-containing membranes, assessed by εCOP-YFP labeling, in dual-color time-lapse experiments in ldlF cells co-expressing cyan fluorescent protein (CFP)-tagged secretory cargo markers. εCOP-YFP was present on juxtanuclear Golgi membranes and on pre-Golgi transport intermediates containing secretory cargo, and it was depleted from retrograde (Golgi-to-ER) transport intermediates. The pre-Golgi intermediates remained brightly labeled with εCOP-YFP as they tracked into the Golgi.

To gain insight into the role of COPI on anterograde (ER-to-Golgi) transport intermediates, we used photobleaching techniques to investigate the characteristics of COPI binding and release from membranes. Upon photobleaching either Golgi or pre-Golgi structures expressing εCOP-GFP in ldlF cells, fluorescence recovered onto these structures exponentially with a half-time of 35 seconds, thus indicating that coatomer binding and dissociation from membranes occur continuously. To test whether the activity is coupled to vesicle budding, we measured the kinetics of coat exchange on and off membranes, at low temperatures, in which vesicle transport is nonexistent. We found that at all temperatures down to 4°C, εCOP-GFP underwent binding and release from Golgi membranes, with no abrupt change in the kinetics on reaching temperatures at which vesicle budding is slowed or inhibited. The data thus indicated that the cycling of coatomer on and off membranes can be uncoupled from vesicle formation and that feedback from productive vesicle budding is not necessary for COPI dissociation. Based on these findings, we proposed that membrane binding and release of COPI serves to initiate and stabilize lateral sorting and segregation of cargo into membrane domains that progressively differentiate into pleiomorphic membrane transport intermediates and/or vesicles for membrane transport in the ER/Golgi system.

### Arf1 Regulation of COPI Dynamics

*Liu, Presley, Pfeffer*

COPI is known to be recruited to membranes by the GTP-binding protein Arf1 while GTP hydrolysis of GTP-bound Arf1 is believed to be necessary for COPI release from membranes. To gain further insight into Arf1 regulation of COPI dynamics, we asked if Arf1 GTP hydrolysis is sufficient for COPI to dissociate from membranes; that is, whether Arf1 and COPI dissociate together from membranes. To address this question, we used a functional Arf1-CFP chimera that allowed us to visualize the behavior of Arf1 and coatomer in the same cell. FRAP experiments revealed that the half-time for Golgi membrane dissociation of Arf1-CFP (13s) was significantly faster than for εCOP-YFP (30s), suggesting that Arf1 and coatomer dissociate independently from Golgi membranes. These results, in turn, were supported by results from experiments using BFA, which inhibits Arf1 and COPI recruitment to membranes, allowing the dissociation of these proteins to be observed in the absence of rebinding. On treatment with BFA. Arf1-CFP dissociated significantly faster ($T_{1/2}$ 13s) than εCOP-YFP ($t_{1/2}$ 30s). The findings indicated therefore that Arf1 and coatomer dissociation from Golgi membranes are regulated differently. A mathematical formulation of COPI and Arf1 membrane binding and dissociation kinetics was able to fit simultaneously both Arf1 and COPI photobleaching data and the Arf1 and COPI release kinetics following BFA treatment, providing new quantitative estimates of the lifetime of these molecules on membranes and their binding and dissociation rates.

## Constitutive Cycling of Golgi Proteins between ER and Golgi

*Ward*

A major controversy in the field of organelle biology has centered on which of two mechanisms, template versus self-organization. best describes the growth and inheritance of the Golgi apparatus. The template model views the Golgi as an autonomous organelle with stable components that provide a template or scaffold for its growth and division. The *de novo* model, by contrast, envisions the Golgi as a dynamic, steady-state structure whose maintenance and biogenesis depend on dynamic membrane input and outflow pathways. A key prediction of the template model is that stably associated proteins on Golgi membranes serve as a scaffold or template for Golgi growth and remodeling. To test such a hypothesis, we used a photobleaching assay to determine whether any Golgi proteins were permanently associated with the Golgi. We tested GFP-tagged members of four different Golgi protein classes (i.e., enzyme, itinerant, matrix, and coat). Our results revealed that none was stably associated with Golgi membranes; after selective photobleaching, fluorescence recovered into the Golgi area in all cases. For GalT-GFP and p58-GFP, which represent the glycosylation enzyme and itinerant classes of Golgi proteins, respectively, recovery occurred on a time scale of minutes and depended on microtubule-driven membrane delivery from the ER. For GRASP65-GFP and εCOP-GFP, which represent the matrix and coat classes of Golgi proteins, repectively, recovery occurred within 20 seconds of Golgi photobleaching and did not depend on the presence of microtubules. Thus, members of all Golgi protein classes localize dynamically to Golgi membranes through either association/dissociation with the cytoplasm or membrane cycling pathways through the ER.

## Building the Golgi *de Novo*

*Ward*

To understand what underlies the ability of the Golgi to maintain and organize itself in the absence of long-lived components, we examined how Golgi structure becomes disorganized under different treatments. The phenotype of Sar1[H79G]-expressing cells (in which Sar1 is locked in its GTP-bound state) was found to resemble that of BFA-treated or Arf1[T31N]-expressing cells (in which Arf1 is locked in its GDP-bound state). Under all three conditions, COPI dissociated from membranes, COPII components continued to exchange on/off ER export sites, and Golgi structure was lost as Golgi enzymes redistributed to the ER and matrix/itinerant proteins relocated to ER export domains. No stable Golgi remnant was found to persist under any of these conditions. Instead, structures containing Golgi matrix and itinerant proteins present in these cells represented ER export domains active for the recruitment of some (including Golgi matrix and itinerant proteins) proteins that populate the secretory pathway.

To test if the complete disruption of ER exit machinery would result in the absorption of all Golgi components into the ER, we expressed the constitutively inactive Sar1[T39N] mutant that is blocked in the GDP-bound state. In these cells, no ER exit sites were visible by EM, and ER export machinery, including Sar1 and Sec13-YFP, were diffusely localized to the ER, which appeared swollen due to accumulated secretory cargo. Golgi matrix proteins, including GRASP65 and GM130, and the recycling protein p58-GFP then redistributed into the ER, where Golgi enzymes were localized. Given that there are no non-ER structures with which Golgi components associate under these conditions, the data demonstrated that there is no stable scaffold or higher order architecture of the Golgi apparatus.

Taken together, our findings suggest that the Golgi apparatus exists as a steady-state system in interphase cells, with rapid and substantial recycling of Golgi proteins through the ER. At all times, a significant fraction of Golgi-resident proteins resides in the ER, available for export into pre-Golgi elements that subsequently become part of the Golgi complex. When such activities are disrupted, the Golgi structure breaks down and disappears within cells. Maintenance of the Golgi complex thus depends on continual membrane export from the ER, export that is controlled by the sequential activities of the Sar1/COPII and Arf1/COPI coat systems.

### Arf1 Regulation of Mitotic Golgi Disassembly

*Altan*

Previous work from our laboratory has shown that during mitosis the Golgi apparatus disperses and reforms through the intermediary of the ER, exploiting constitutive ER/Golgi recycling pathways. Over the past year, we have focused on understanding how and why this occurs. An important clue for addressing these questions came with our finding that treatment of cells with the drug H89, known to prevent Golgi disassembly caused by BFA-induced Arf1 inactivation, also blocks mitotic Golgi disassembly. This finding led us to focus on Arf1, the target of BFA, as a regulator of mitotic Golgi disassembly. Live cell imaging of a single cell expressing Arf1-CFP showed that Arf1 dissociated from Golgi membranes and became cytoplasmic in prophase before Golgi fragmentation. Addition of H89 completely blocked Arf1 release from Golgi membranes. We observed a similar phenotype in cells expressing Arf1[Q71L], which cannot undergo GTP hydrolysis and release from Golgi membranes. Our observation suggested that both mitotic and BFA-induced Golgi breakdown depend on Arf1 inactivation. We used FRAP both to analyze the mechanism for Arf1 inactivation in mitotic cells and to measure the binding/dissociation properties of Arf1 in interphase and prophase cells. Our results suggested that Arf1 inactivation and depletion from Golgi membranes in BFA-treated and mitotic cells occurs through a common mechanism: the inhibition of Arf1-GEF. The results imply that Arf1 regulates Golgi structure in both interphase and mitosis.

## SELECTED PUBLICATIONS

1. Aridor M, Fish KN, Bannykh S, Weissman J, Roberts TH, Lippincott-Schwartz J, Balch WE. The Sar1 GTPase coordinates biosynthetic cargo selection with endoplasmic reticulum export site assembly. J Cell Biol. 2001;152:1-18.
2. Brandizzi F, Snapp E, Roberts A, Lippincott-Schwartz J, Hawes C. Membrane protein transport between the ER and Golgi in tobacco leaves is energy dependent but cytoskeleton independent: evidence from selective photobleaching. Plant Cell. 2002;14:1293-1309.
3. Daigle N, Beaudouin J, Hartnell L, Imreh G, Hallberg E, Lippincott-Schwartz J, Ellenberg J. Nuclear pore complexes form immobile networks and have a very low turnover in live mammalian cells. J Cell Biol. 2001;154:71-84.
4. Griffis E, Altan N, Lippincott-Schwartz J, Powers M. Nup98 is a mobile nucleoporin with transcription dependent dynamics. Mol Cell Biol. 2002;13:1282-1297.
5. Lippincott-Schwartz J. The endoplasmic reticulum and Golgi complex in secretory membrane transport. The liver: biology and pathobiology, 4th ed. New York: Raven Press; in press.
6. Lippincott-Schwartz J. The secretory membrane system studied in real-time. Histochem Cell Biol. 2001;116:97-107.
7. Lippincott-Schwartz J. Tearing up the nuclear envelope. Nature. 2002;416:31-32.
8. Lippincott-Schwartz J, Snapp E, Kenworthy A. Studying protein dynamics in living cells. Nat Cell Biol Rev. 2001;2:444-456.

9. Nichols BJ, Kenworthy AK, Roberts TH, Hirschberg K, Lodge R, Phair RD, Lippincott-Schwartz J. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. J Cell Biol. 2001;153:529-541.
10. Nichols B, Lippincott-Schwartz J. Endocytosis without clathrin coats. Trends Cell Biol. 2001;11:406-412.
11. Nikonov AV, Snapp E, Lippincott-Schwartz J, Kreibich G. Active translocon complexes diffuse slowly in the membrane of the ER and behave as large polysome arrays. J Cell Biol. 2002;158:497-506.
12. Patterson G, Lippincott-Schwartz J. A photoactivatable GFP for selective photolabeling of proteins and cells. Science. 2002;297:1873-1877.
13. Presley JP, Ward T, Miller C, Siggia E, Phair RD, Lippincott-Schwartz J. Dissection of COPI and Arf1 dynamics *in vivo* and role in Golgi membrane transport. Nature. 2002;417:187-193.
14. Rotman-Pikienlny P, Hirschberg K, Maruvada P, Suzuki K, Royaux I, Green E, Kohn L, Lippincott-Schwartz J, Yen PM. Retention of pendrin in the endoplasmic reticulum is a major mechanism for Pendred Syndrome. Human Molec Genet. 2002;11:1-9.
15. Ward T, Polishchuk R, Hirschberg K, Barr F, Lippincott-Schwartz J. Maintenance of Golgi structure and function depends on the integrity of ER export. J Cell Biol. 2001;155:557-570.
16. Yamazaki T, Zaal K, Hailey D, Presley J, Lippincott-Schwartz J, Samelson L. Role of Grb2 in EGF-stimulated EGFR internalization. J Cell Sci. 2002;115:1791-1802.

## COLLABORATORS

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# REGULATION OF INTRACELLULAR IRON METABOLISM

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Previously, our laboratory identified and characterized the *cis* and *trans* elements that regulate iron-dependent alterations in expression of ferritin and the transferrin receptor. Iron-responsive elements (IREs) are RNA stem-loops found in the 5' end of ferritin mRNA and the 3' end of transferrin receptor mRNA. We have cloned, expressed, and characterized two essential iron-sensing proteins, Iron Regulatory Protein 1 (IRP1) and Iron Regulatory Protein 2 (IRP2). IRPs bind to IREs when iron levels are depleted, resulting in both the inhibition of translation of ferritin mRNA and other transcripts with IREs near the 5' end and the prolongation of the half-life of the transferrin receptor mRNA.

## Iron-Sulfur Cluster Assembly

*Tong*

IRP1 is an iron-sulfur protein related to mitochondrial aconitase, a citric acid cycle enzyme that functions as a cytosolic aconitase in cells that are iron-replete. Regulation of the RNA binding activity of IRP1 involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound to a form that loses both iron and aconitase activity. The [4Fe-4S]–containing protein does not bind to IREs, and the status of the cluster appears to determine whether IRP1 will bind to RNA. Recently, we have identified mammalian enzymes of iron-sulfur cluster assembly that are homologous to the NifS and Nif U genes implicated in bacterial iron-sulfur cluster assembly. We have shown that these gene products facilitate assembly of the iron-sulfur cluster of IRP1. We discovered that single genes in the human genome encode both mitochondrial and cytosolic forms of the cysteine desulfurase IscS, IscU, and NFU. NFU is abundant and may represent a scaffold for iron-sulfur cluster assembly.

## Iron-Dependent Degradation of IRP2 and Other Proteins

*Ghosh*

IRP2 also binds to IREs in iron-depleted cells, but unlike IRP1, IRP2 is degraded in cells that are replete with iron. Experimental evidence indicates that IRP2 undergoes iron-catalyzed oxidation. The proteasome then selectively ubiquitinates and degrades the oxidized protein. Indirect evidence suggests that numerous other proteins are degraded by a pathway in which oxidative modification is followed by ubiquitination and proteasomal degradation of the ubiquitinated substrate.

## Physiology and Regulation of Iron Metabolism

*Smith, Cooperman, Grabill, Jui-Chen, Meyron-Holtz, Land, Missirlis*

To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we observed no abnormalities in iron metabolism associated with loss of IRP1 function. IRP2-/- mice develop a progressive movement disorder characterized by gait abnormalities and tremor. Animals accumulate iron in axons and develop axonal degeneration. Ferritin over-expression occurs in affected neurons, and ferritin accumulates in axons. Affected tissues are those that contain cells that depend mainly on IRP2 for repression of ferritin translation. Animals that lack both IRP1 and IRP2 do not survive past the blastocyst stage.

### Structural Characterization of IRPs and IREs

*Yikilmaz*

We have purified milligram quantities of IRP1 and IRP2 and are working on crystallization of each IRP. In addition, we are trying to co-crystallize each IRP in a complex with IRE. We have characterized and over-expressed an IRP-like protein from *Plasmodium falciparum*. We have also characterized an IRE in the 3'UTR of the iron transporter DMT1 that binds with high affinity to only one of the two IREs.

## SELECTED PUBLICATIONS

1. Bolan CD, Conry-Cantilena C, Mason G, Rouault TA, Leitman SF. MCV as a guide to phlebotomy therapy for hemochromatosis. Transfusion. 2001;41:819-827.
2. Drake SK, Bourdon E, Wehr N, Levine RL, Backlund P, Yergey A, Rouault TA. Numerous proteins in mammalian cells are prone to iron-dependent oxidation and proteasomal degradation. Dev Neurosci. 2002;24:114-124.
3. Gunshin H, Allerson CR, Polycarpou-Schwarz M, Rofts A, Rogers JT, Kishi F, Hentze MW, Rouault TA, Andrews NC, Hediger MA. Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett. 2001;509:309-316.
4. LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, Drake SK, Miller G, Abu-Asab M, Tsokos M, Switzer R, Grinberg A, Love P, Tresser N, Rouault TA. Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. Nat Genet. 2001;27:209-214.
5. Loyevsky M, LaVaute T, Allerson CR, Stearman R, Kassim OO, Cooperman S, Gordeuk VR, Rouault TA. An IRP-like protein from Plasmodium falciparum binds to a mammalian iron-responsive element. Blood. 2001;98:2555-2562.
6. Rouault TA. Iron on the brain. Nat Genet. 2001;28:299-300.
7. Rouault TA. Systemic iron metabolism: a review and implications for brain iron metabolism. Pediatr Neurol. 2001;25:130-137.
8. Tong WH, Rouault TA. Evidence for two distinct iron-sulfur cluster assembly complexes in the cytosol and in the mitochondria of mammalian cells. EMBO J. 2000;19:5692-5700.

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# GENDER-SPECIFIC BRAIN AND REPRODUCTIVE DEVELOPMENT

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Judith Ross, M.D., *Guest Researcher*[c]
Andrew Zinn, M.D., Ph.D., *Guest Researcher*[d]

The Sections on Growth and Metabolism and Women's Health integrate basic and clinical research to elucidate genetic and physiological mechanisms governing male and female development. We are particularly interested in identifying X-chromosome genes involved in sexually dimorphic development of the reproductive systems and brain and in distinguishing these effects from those of steroid hormones.

## Growth Factors, Hormones, and Brain Development

*Cheng, Chen, Wang, Smith, Bondy*

The growing brain consumes about 50 percent of the total fuel available to the organism as a whole during early postnatal development, despite the fact that its mass is less than 10 percent of the whole. How the brain competes so successfully with peripheral tissues for energy and substrates has been unclear. Insulin preferentially enhances fuel use by peripheral tissues, but not by the brain. We previously demonstrated that endogenous brain IGF1 serves an insulin-like role in promoting neuronal glucose utilization and hence growth during postnatal development. We have shown that brain growth in *Igf1* null mice falls behind that of normal littermates by almost 40 percent during the postnatal period, which is when brain IGF1 expression is normally most abundant. We have also demonstrated that brain glucose uptake and utilization is profoundly reduced in the *Igf1* null brain during this period. Our studies have implicated IGF1-induced phosphorylation of Akt/PKB in translocation of glucose across the neuronal membrane and IGF1-induced phosphorylation of GSK3b in neuronal glycogenesis, suggesting that IGF1 augments neuronal glucose uptake and storage by familiar, insulin-like pathways. Over the past year, we have investigated the consequences of IGF1 deletion for neuronal survival and growth/morphogenesis.

While cell numbers were preserved throughout most brain structures in the *Igf1* null brain, there was a significant reduction in dentate granule cell number in the hippocampal formation, a structure critical for memory. Cell proliferation was actually either normal or increased in the *Igf1* null dentate germinal zone, but cell death increased even more, explaining the reduction in dentate size in the IGF1's absence. Neuronal numbers were preserved, but morphometric analysis showed that pyramidal neuron soma size decreased by about 10 percent in the *Igf1* null frontoparietal cortex. Golgi staining revealed that cortical neurons exhibited a significant reduction in dendritic length and complexity in *Igf1* null mice. In addition, the density of dendritic spines and, presumably, synaptic contacts declined by 16 percent in the *Igf1* null brain ( Fig. 2). Synaptotagmin levels decreased by 30 percent (P = 0.01) in the *Igf1* null brain, yielding evidence in support of a reduction in synapses.

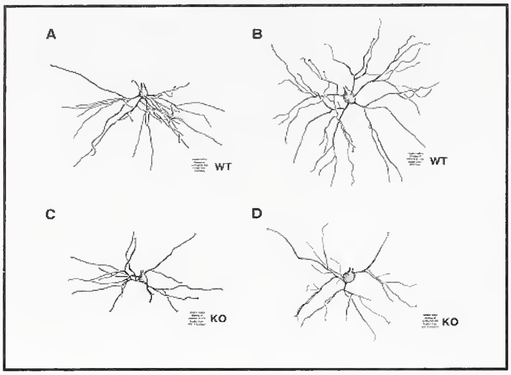

**FIGURE 2**

Camera lucida drawings of Golgi-stained layer 2–3 pyramids. A & B are wild type (WT) and C & D are knockout (KO) from Igf1 null brains.

We have proposed that IGF1's primary physiological role in normal brain development is to promote the growth of projection neurons. IGF1 expression is most abundant in neurons (e.g., Purkinje cells) destined to be the largest and most complex in the brain, implicating IGF1's insulin-like, anabolic effects in this extraordinary growth. IGF1-expressing neurons grow large perikarya, long axons, and extraordinarily prolific dendritic arbors, which are severely hypoplastic in the *IGF1* null brain. Homozygous *IGF1* deletion results in mental retardation, showing that the effects of this anabolic peptide on neuronal metabolism and growth have important implications for cognitive function. Current work in our laboratory is aimed at discovering factors regulating neuronal IGF1 expression with a view to therapeutic manipulation to increase the potential for cognitive development and for successful responses to brain injury.

## Estrogen Regulation of Gene Expression in the Primate Neocortex

*Cheng, Wang, Smith, Bondy*

Estrogen has well-established neurotrophic effects *in vitro* and is reputed to protect against Alzheimer's disease in post-menopausal women. To elucidate the molecular basis for these neuroprotective effects, we have investigated the effects of estradiol (E2) versus placebo on brain gene expression in a primate model system: ovariectomized rhesus monkeys. We previously showed that E2 enhances the expression of glucose transporters and IGF1 in the monkey prefrontal cortex, a brain region involved in higher-level cognitive functioning, thus suggesting a role for IGF1 and augmented glucose transport in estrogen's salutary effects on brain function. Using DNA array technology, studies over the past year have focused on profiling a larger spectrum of cortical genes regulated by estrogen. E2 treatment produced significant alterations in gene expression profiles in the prefrontal cortex. The levels of 61 transcripts increased while those of 77 decreased more than 1.5-fold in the E2-treated group, with greatest effects including an approximately four-fold increase in the ribosomal protein S6 kinase and a 4.3-fold decrease in malate dehydrogenase 1. About 15 percent of the altered transcripts encode metabolic enzymes, 12 percent transcription factors, 12 percent cytoskeletal proteins, and 10 percent protein kinases, with the remainder belonging to diverse functional categories. Fig. 3 shows confirmation of some of these changes at the protein level. The findings show robust and complex genomic effects by E2 on a brain structure that is of major

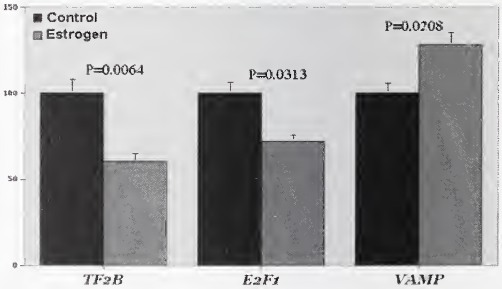

**FIGURE 3**

Estrogen decreases the level of transcription factors TF2B and E2F1 and increases the expression of the synaptic protein VAMP in the primate prefrontal cortex. Data from immunoblots of protein extracted from estrogen and placebo-treated monkeys (n=4–5/group).

importance for higher cognitive functions. Identification of cell types involved in these changes and investigation of potential functional interrelationships between the altered gene products should elucidate E2's role in cognitive function and neuroprotection.

### Effects of X-Chromosome Deletion on Bone Fragility and Intermediary Metabolism

*Bakalov, Hanton, Luo, Zinn, Bondy*

Turner Syndrome (TS) involves the loss of all or part of one sex chromosome and is observed in one out of 2,500 live female births. We have recently initiated genotype-phenotype studies aimed at identifying X-chromosome genes responsible for abnormalities in development and function of the brain, cardiovascular system, and ovary in TS. The new studies are also focusing on the clinical characterization and genetic tracking of metabolic disorders, including osteoporosis, glucose intolerance, hypertension, and dyslipidemia, which appear to affect greater-than-expected numbers of individuals with TS. The short stature typical of the disorder is thought to be due primarily to haplo-insufficiency for the *SHOX* gene product. Several studies have reported an increased prevalence of bone fractures in TS, which has been attributed either to *SHOX* deletion or to estrogen insufficiency owing to ovarian failure. We have shown that, when fracture history is carefully documented through personal interviews with both subjects and age-matched controls, there is no excess of fractures in TS. Moreover, we have demonstrated that bone mineral density, when measured by using volumetric methods not dependent on bone size, is virtually normal in TS women on conventional hormone replacement treatment. This analysis appears to exclude a role for the *SHOX* gene product in preventing osteoporosis or bone fragility.

Reports on diabetes mellitus (DM) in TS have varied widely, with some studies indicating prevalence up to 40 percent and others finding no increase over normal populations (about 5 percent). We have now shown that, while most girls and women with TS have normal fasting glucose and insulin, the response to a glucose challenge is markedly abnormal in at least 40 percent of lean young women after exclusion of obese subjects from the analysis. Multivariate analyses have also excluded significant contributions of family history and ovarian failure to this diabetic phenotype. Preliminary data suggest a primary insulin secretory defect in young lean women and girls with TS, without evidence of insulin resistance as seen in typical type 2 DM. Since about 40 percent of the group is affected and thus 40 percent gets its single normal X from the father, we hypothesize that a gene promoting pancreatic insulin synthesis/secretion is selectively active on the maternal X but imprinted or silent on the paternal X chromosome. Genotyping of parental chromosomes will allow testing of this hypothesis, and evaluation of subjects with partial X deletions will allow us to search for specific genes responsible for this metabolic phenotype.

### Role of Ovarian Androgens in Protection of the Mammary Gland

*Zhou, Dimitrakakis, Bondy*

Androgens are normally quite abundant in healthy women; indeed, they are relatively more abundant than estrogens throughout the entire life cycle. Androgens' normal physiological role in women is thought to involve augmentation of lean body mass, energy, and libido. We have proposed that another important role for endogenous androgen in women is to protect the mammary gland from "unopposed" estrogenic stimulation. We have shown that treatment of normally cycling rhesus monkeys with flutamide, an androgen receptor antagonist, enhances mammary epithelial proliferation by about 50 percent, indicating that androgen receptor activation normally suppresses mammary epithelial proliferation. To evaluate the efficacy of physiologic androgen supplementation in limiting estrogen replacement therapy–induced mammary epithelial proliferation, we employed an ovariectomized rhesus monkey model of menopause. Proliferation was increased about four-fold in the groups treated with estradiol and estradiol plus progesterone but was no different from vehicle-treated control in the group on estradiol plus testosterone. These observations suggest that endogenous androgens normally limit mammary epithelial proliferation and that androgen supplementation of estrogen therapy may reduce estrogen-induced proliferation and breast cancer risk. Potentially explaining at least partially the mechanism of testosterone's protective effects, we have shown that estrogen receptor (ER) alpha is down-regulated and ER beta up-regulated by testosterone. Since the alpha isoform mediates proliferative effects and *MYC* expression, while the beta isoform does not, the dramatic alteration in ER alpha/beta ratio may be integral to testosterone's inhibition of estrogen-induced proliferation. Indeed, we have also demonstrated a significant reduction in *MYC* expression in the estrogen/testosterone group (Fig. 4).

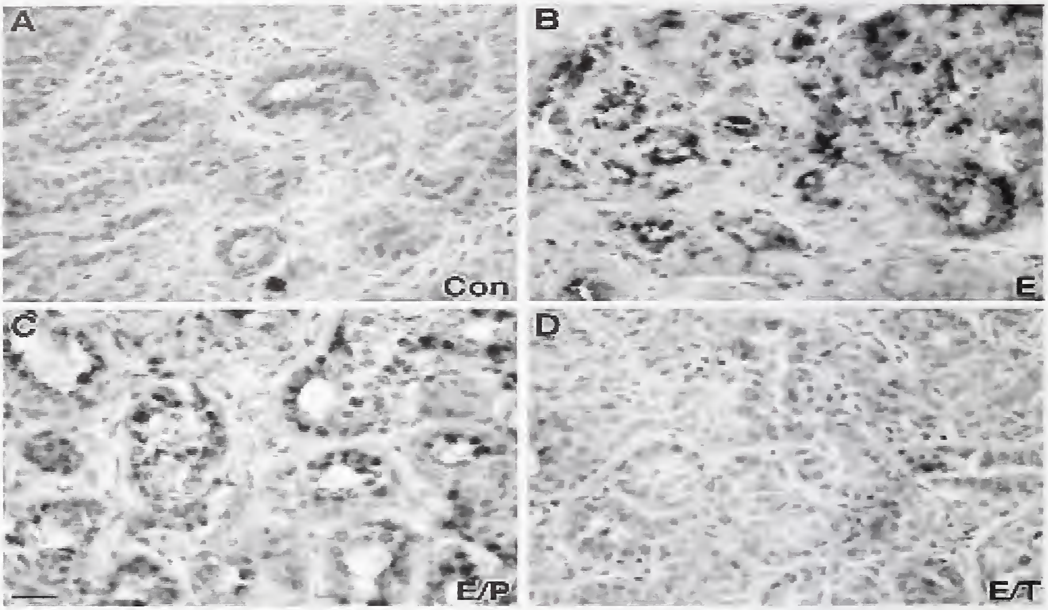

**FIGURE 4**

Estrogen (E) and estrogen plus progesterone (E/P) strongly induce MYC expression in the primate mammary epithelium while estrogen plus testosterone (E/T) do not; compared with control (Con).

## SELECTED PUBLICATIONS

1. Arraztoa JA, Bondy C, Monget P, Zhou J. Expression patterns of insulin-like growth factor binding proteins 1, 2, 3 and 5 in the non-human primate ovary. J Clin Endocrinol Metab. 2002;87:5220-5228.
2. Bakalov VK, Vanderhoof VH, Bondy CA, Nelson LM. Adrenal antibodies detect asymptomatic autoimmune adrenal insufficiency in young women with spontaneous premature ovarian failure. Hum Reprod. 2002;17:903-911.
3. Cheng C, Cohen M, Tseng V, Bondy C. Endogenous IGF1 enhances cell survival in the postnatal dentate gyrus. J Neurosci Res. 2001;64:341-347.
4. Cheng, C, Cohen M, Wang J, Bondy CA. Estrogen augments glucose transporter and IGF1 expression in primate cerebral cortex. FASEB J. 2001;15:907-915
5. Cheng C, Reinhardt RR, Lee W-H, Joncas G, Patel SC, Bondy CA. IGF1 regulates developing brain glucose metabolism. Proc Natl Acad Sci U S A. 2000;97:10236-10241.
6. Dimtrakis C, Zhou J, Bondy CA. Androgens, mammary growth and neoplasia. Fertil Steril. 2002;77:26-33.
7. Kadakia R, Arraztoa JA, Bondy C, Zhou J. Granulosa cell proliferation is impaired in the Igf1 null ovary. Growth Horm IGF Res. 2001;11:220-224.
8. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. Endocr Rev. 2001;22:53-74.

9. Monget P, Bondy C. Importance of the IGF system in early folliculogenesis. Mol Cell Endocrinol. 2000;163:89-93.

10. Ross JL, Stefanatos GA, Kushner H, Zinn A, Bondy C, Roeltgen D. Persistent cognitive deficits in adult women with Turner syndrome. Neurology. 2002;58:218-225.

11. Tong ZB, Bondy CA, Zhou J, Nelson LM. A human homolog of mouse mater, a maternal effect gene essential for early embryonic development. J Hum Reprod. 2002;17:903-911.

12. Tong ZB, Gold L, Pfeifer KE, Dorward H, Lee E, Bondy CA, Dean J, Nelson LM. Mater, a maternal effect gene required for early embryonic development in mice. Nat Genet. 2000;26:267-268.

13. Valencia JC, Matsui K, Bondy C, Zhou J, Rasmussen A, Cullen K, Yu ZX, Moss J, Ferrans VJ. Distribution and mRNA expression of insulin-like growth factor system in pulmonary lymphangioleiomyomatosis. J Investig Med. 2001;49:421-433.

14. Zhou J, Anderson K, Bievre M, Ng S, Bondy CA. The primate mammary gland insulin-like growth factor system: cellular localization and regulation by sex steroids. J Investig Med. 2001;49:47-55.

15. Zhou J, Bievre M, Bondy CA. Reduced GLUT1 expression in Igf1-/- null oocytes and follicles. Growth Horm IGF Res. 2000;10:111-117.

16. Zhou J, Ng S, Adesanya-Famuiya O, Anderson K, Bondy CA. Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression. FASEB J. 2000;14:1725-1730.

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# MOLECULAR GENETICS OF ADRENOCORTICAL TUMORS AND RELATED DISORDERS

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Audrey Robinson-White, Ph.D., *Guest Researcher*

The goal of our work is to understand the genetic and molecular mechanisms leading to disorders that affect the adrenal cortex, with emphasis on those that are developmental, hereditary, and associated with adrenal hypoplasia or hyperplasia, multiple tumors, and abnormalities in other endocrine glands, such as the pituitary gland. In this context, our laboratory has studied congenital adrenal hypoplasia caused by Allgrove syndrome (or triple A syndrome) and multiple endocrine deficiencies (APECED syndrome), familial hyperaldosteronism, adrenocortical and thyroid cancer, pituitary tumors, multiple endocrine neoplasia syndromes (MEN 1, Cowden disease) affecting the pituitary, thyroid, and adrenal glands, and Carney complex, an autosomal dominant disease that affects the adrenal cortex. Recently, our laboratory identified the regulatory subunit type 1-α(RIαof protein kinase A (PKA) that is encoded by the *PRKAR1A* gene, the gene responsible for almost half of the patients with Carney complex. Since then, our laboratory has focused on PKA-stimulated signalling pathways, PKA effects on tumor suppression and/or development, the cell cycle and chromosomal stability, and PKA-dependent hormonal interactions.

### Carney Complex Genetics

*Bauer, Bei, Bourdeau, Griffin, Matyakhina, Stratakis; in collaboration with Carney, Kirschner, Bertherat*

Carney complex is the only inherited form of adrenal gland-dependent Cushing's syndrome; it is also a multiple endocrine neoplasia affecting the pituitary, thyroid, and gonads, and it is associated with a variety of other tumors, including myxomas (heart and other myxomas) and schwannomas (it is one of three conditions associated with inherited schwannomas; the other two are neurofibromatosis and isolated schwannomatosis), and with skin pigmentation defects (lentigines, café-au-lait spots, and nevi).

Families with Carney complex (CNC) and related syndromes have been collected from a number of collaborating institutions worldwide. Genetic linkage analysis identified loci harboring genes for CNC on chromosomes 2 (2p16) and 17 (17q22-24); possible other loci for this genetically heterogeneous condition are currently under investigation by using state-of-the-art molecular cytogenetic techniques. We have constructed a comprehensive genetic and physical map of the 2p16 chromosomal region for the cloning of the CNC-associated sequences from this region. Studies in cultured primary tumor cell lines (established from our patients) identified a region of genomic instability in the center of the map (Fig. 5). Tumor studies led to the identification of the *PRKAR1A* gene on 17q22-24 as the gene responsible for CNC in approximately 40 percent of the cases of the

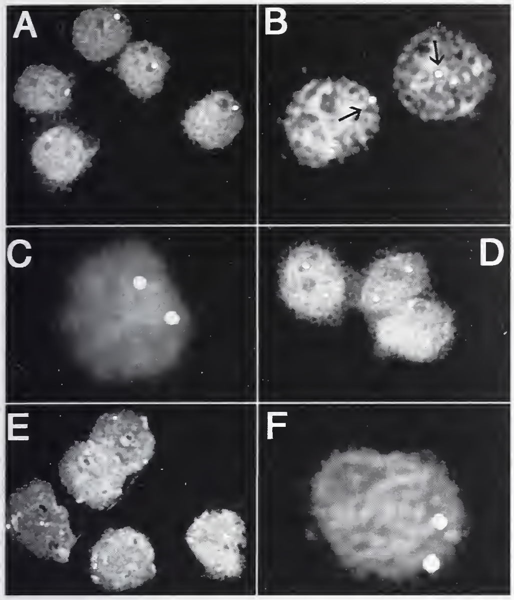

**FIGURE 5**

Deletions of the 17q22-24 *PRKAR1A* region in sproradic adrenal tumors. (A) Interphase FISH with the BAC 321-G-8 containing the *PRKARIA* gene in an adrenal adenoma showed cells with one signal of the BAC (white spots). (B) FISH with the BAC 321-G-8 (white spots) and an a-satellite probe specific for chromosome 17 (white spots with arrow) to an adrenal carcinoma showed cells with one signal for each probe suggesting loss of chromosome 17 from the centromere to the 17q22-24 region. (C) A control interphase FISH for the same carcinoma with BAC RP3-526I14 from chromosome 22 showed the expected two signals. (D) Likewise, FISH with BAC 588-h-15 from chromosome 6 (white spots) to an adenoma that showed loss for 17q22-24, showed the expected two signals. (E) Normal adrenal cells hybridized with BAC 321-G-8 showed two copies of the probe. (F) Hybridization of BAC 321-G-8 (harboring the PRKAR1A gene) to another carcinoma detected two copies of the probe in all cells showing that this particular tumor had not undergone losses of the 17q22-24 region; these data were consistent with the LOH (by polymorphic markers) analysis for this tumor.

disease. It was determined that *PRKAR1A* functions as a novel tumor suppressor gene in CNC-associated tumors; it is also the main regulatory subunit of protein kinase A (PKA), a central signalling pathway for many of cellular functions and hormonal responses. More patients with CNC are now the subject of genotype-phenotype correlation studies, which are expected to shed light into the complex biochemical and molecular pathways regulated by *PRKAR1A* and PKA.

### Effects of *PRKAR1A* on Protein Kinase A Activity and Endocrine Tumor Development

*Matyakhina, Sandrini, Stergiopoulos, Robinson-White, Stratakis; in collaboration Cho-Chung, Bertherat*

The functional consequences of *PRKAR1A* mutations are under investigation in cell lines established from CNC patients and their tumors. Both cAMP and PKA activity are measured in the cell lines, along with the expression of the other subunits of the PKA tetramer. We have established stable transfectants of antisense *PRKAR1A* constructs in commercially available mouse endocrine and other cell lines; we study the cell lines for the effects of *PRKAR1A* silencing on their growth, differentiation, and proliferation. We hypothesize that the tumorigenicity of *PRKAR1A*-inactivating mutations relies on a switch from type-I PKA (based almost exclusively on endocrine cells on PRKAR1A) to type-II PKA activity; cell lines with an antisense *PRKAR1A* construct are believed to be a representative model of the *in vivo* situation in CNC patients. In addition to the above, we are seeking mutations of the *PRKAR1A* gene, which would further establish the gene's role as a general tumor suppressor, in sporadic endocrine and non-endocrine tumors (thyroid adenomas and carcinomas, adreno-cortical adenomas and carcinomas, ovarian carcinomas, melanomas and other benign and malignant pigmented lesions, and heart myxomas). We receive specimens on a collaborative basis from a variety of investigators within the NIH and around the world [Dr. Sarlis (NIDDK), Dr. Eng (Ohio State University), Dr. Melmed (UCLA), Dr. Tanaka (Tokio University), Dr. Lacroix (University of Montreal)].

### Animal Models of *PRKAR1A*

*Bauer, Griffin, Claflin, Shiferaw, Stratakis; in collaboration with Westphal, Kirschner*

The *PRKAR1A*-knock-out (KO) (-/-) mouse, created several years ago by S. McKnight of the University of Washington, Seattle, dies of heart and central nervous system abnormalities on day nine of embryonic development. Since the discovery of *PRKAR1A*'s involvement in CNC, our laboratory has been developing conditional *PRKAR1A* KOs in endocrine tissues (adrenal cortex, anterior lobe of the pituitary, and thyroid). The first analysis of a one-year-old mouse expressing an antisense *PRKAR1A* transgene is ongoing.

### *PRKAR1A*, the Cell Cycle, Chromosomal Stability, and Other Signalling Pathways

*Matyakhina, Shiferaw, Robinson-White, Stratakis; in collaboration with Papadopoulos*

Genes implicated in cyclic nucleotide–dependent signalling have long been considered likely candidates for endocrine tumorigenesis. Somatic activating mutations in a number of G protein–coupled receptors (GPCRs) and the gene encoding a subunit of the stimulatory G protein (*GNAS1*) lead to increased cAMP production and are responsible for a number of endocrine tumors of various types. To date, however, there is no convincing evidence that, in the absence of additional genetic abnormalities, GNAS1 or GPCR activation is involved in cancer. Individuals with McCune-Albright syndrome (MAS) who bear somatic GNAS1 mutations in their endocrine glands may be predisposed to developing some cancers (MAS exhibits similarities to CNC). However, activation of additional pathways and/or other changes appear to be required for the *in vitro* transformation of 3T3 or FRTL5 cells by constitutively active GPCR transgenes or in other settings of increased cAMP signalling that lead to malignant transformation. Thus, other genes that regulate PKA function and increase cAMP-dependent proliferation and related signals may be altered in the process of endocrine tumorigenesis initiated by a mutant *PRKAR1A*, a gene with functions important to both the cell cycle and chromosomal stability. Our work aims to identify these interactions of *PRKAR1A* by studying mitogenic and other growth-signalling pathways in cell lines expressing the antisense *PRKAR1A* constructs referred to above. In addition, chromosomal stability in both human and mouse cell lines in which *PRKAR1A* has been inactivated is under investigation with classic and molecular cytogenetics, including fluorescent *in situ* hybridization (FISH), spectral karyotyping (SKY), and comparative genomic hybridization (CGH). More recently, we have also been investigating proteins that are directly bound to *PRKAR1A* (RIα) and regulate its function; we are particularly interested in the novel protein PAP7, which was identified by a collaborating laboratory. Compartmentalization of PKA function is thought to be mediated by anchoring proteins; almost all the PKA-anchoring proteins known to date, however, are bound to type II-PKA. PAP7 may be the first PKA type-I–specific anchoring protein.

### Genetic Investigations on Other Adrenocortical Diseases and Tumors

*Bourdeau, Sandrini, Farmakidis,* Stratakis; in collaboration with Libutti, Chan, Carney, Stowasser, Torpy, Lacroix, Bertherat*

The goal of our work is to apply general and pathway-specific microarrays to a variety of adrenocortical tumors in order to identify genes with important functions in adrenal oncogenetics; to examine specific candidate genes (such as *TP53* and other tumor suppressors and oncogenes) for their roles in adrenocortical tumors and development; and to identify by positional cloning additional genes with a role in inherited adrenocortical diseases. We undertake a large part of our work collaboratively, as is evident from the accompanying list of collaborating investigators and institutions. As part of this work, we have recently accomplished the following: identification of a novel *TP53* mutation in a cohort of adrenocortical tumors from Southern Brazil with important implications for p53's function in adrenocortical tumor suppression; completion of a genome-wide screen for the identification of gene(s) responsible for inherited adrenocortical aldosteronomas (familial hyperaldosteronism type II - FH-II) that led to the specification of a locus for FH-II on chromosome 7 (7p22); initiation of a genome-wide screen for the identification of a syndrome composed of familial paragangliomas and adrenal, gastric stromal, and pulmonary tumors; and identification of new mutations in the APECED and Allgrove syndrome genes leading to congenital adrenal hypoplasia.

### Genetic Investigations on Other Endocrine Neoplasias and Related Syndromes

*Bauer, Bourdeau, Francis, Sandrini, Stratakis; in collaboration with Marx, Haddad, Blancato, Meck, Eng*

We are also working, largely collaboratively, with a number of other investigators at the NIH and elsewhere on the genetics of CNC- and adrenal-related endocrine tumors, including childhood adrenocortical cancer and thyroid and pituitary tumors. As part of this work, we described novel genetic abnormalities in thyroid tumors. We are also identifying the genetic defects in patients with CNC-related syndromes (the lentigenoses, i.e., Peutz-Jeghers syndrome and others).

### Clinical Investigations in the Diagnosis and Treatment of Adrenal and Pituitary Tumors

*Griffin, Bourdeau, Stratakis; in collaboration with Keil, Patronas*

Ongoing investigations of NIH Clinical Center patients with adrenal tumors and other types of Cushing's syndrome (and occasionally other pituitary tumors) are studying the prevalence of ectopic hormone receptor expression in adrenal adenomas and massive macronodular adrenocortical disease; the diagnostic use of high-sensitivity magnetic resonance imaging for the earlier detection of pituitary tumors; and the diagnosis, management, and post-operative care of children with Cushing's disease and other pituitary tumors.

## SELECTED PUBLICATIONS

1. Bauer AJ, Cavalli LR, Rone JD, Francis GL, Burch HB, Tuttle RM, Ringel MD, Stratakis CA, Haddad BR. Evaluation of adult papillary thyroid carcinomas by comparative genomic hybridization and microsatellite instability analysis. Cancer Genet Cytogenet. 2002;135:182-186.
2. Bourdeau I, Stratakis CA. Cyclic AMP-dependent signaling aberrations in macronodular adrenal disease. Ann NY Acad Sci. 2002;968:240-255.
3. Carney JA, Stratakis CA. Familial paraganglioma and gastric stromal sarcoma: a new syndrome distinct from the Carney triad. Am J Med Genet. 2002;108:132-139.
4. Glasow A, Horn LC, Taymans SE, Stratakis CA, Kelly PA, Kohler U, Gillespie J, Vonderhaar BK, Bornstein SR. Mutational analysis of the prolactin receptor (*PRLR*) gene in human breast tumors with differential PRLR protein expression. J Clin Encocrinol Metab. 2001;86:3826-3832.
5. Groussin L, Kirschner LS, Vincent-Dejean C, Perlemoine K, Jullian E, Delmer B, Zacharieva S, Pignatelli D, Carney JA, Luton JP, Bertagna X, Stratakis CA, Bertherat J. Molecular analysis of the cyclic AMP-dependent protein kinase A (PKA) regulatory subunit 1A (*PRKAR1A*) gene in patients with Carney complex and/or primary pigmented nodular adrenocortical disease (PPNAD) reveals novel mutations and clues for pathophysiology: augmented PKA signaling is associated with adrenal tumorigenesis in PPNAD. Am J Hum Genet. 2002;71:1433-1442.
6. Kaltsas GA, Kola B, Borboli N, Guerguiev M, Swords FM, Czirjak S, Kirschner LS, Stratakis CA, Kobonitz M, Grossman AB. Sequence analysis of the PRKAR1A gene in sporadic somatotroph and other pituitary tumours. Clin Endocrinol (Oxf). 2002;57:443-448.
7. Matyakhina L, Lenherr SM, Stratakis CA. Protein kinase A and chromosomal stability. Ann NY Acad Sci. 2002;968:148-157.
8. Ribeiro RC, Sandrini F, Figueiredo B, Zambetti GP, Michalkiewicz E, Lafferty AR, DeLacerda L, Rabin M, Cadwell C, Sampaio G, Cat I, Stratakis CA, Sandrini R. An inherited p53 mutation that contributes in a tissue-specific manner to pediatric adrenal cortical carcinoma. Proc Natl Acad Sci USA. 2001;98:9330-9335.
9. Robinson-White A, Stratakis CA. Protein kinase A signaling: "cross-talk" with other pathways in endocrine cells. Ann NY Acad Sci. 2002;968:256-270.
10. Sandrini F, Farmakidis C, Kirschner LS, Wu SM, Tullio-Pelet A, Lyonnet S, Metzger DL, Bourdony CJ, Tiosano D, Chan WY, Stratakis CA. Spectrum of mutations of the AAAS gene in Allgrove syndrome: lack of mutations in six kindreds with isolated resistance to corticotropin. J Clin Endocrinol Metab. 2001;86:5433-5437.
11. Sandrini F, Matyakhina L, Sarlis NJ, Farmakidis C, Kirschner LS, Gimm O, Stratakis CA. Regulatory subunit type I-alpha of protein kinase A (*PRKAR1A*): a tumor-suppressor gene for sporadic thyroid cancer. Genes Chromosomes Cancer. 2002;35:182-192.

12. Stratakis CA. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit (PRKAR1A) in patients with the "complex of spotty skin pigmentation, myxomas, endocrine overactivity, and schwannomas" (Carney complex). Ann NY Acad Sci. 2002;968:3-21.
13. Stratakis CA, Cho-Chung YS. Protein kinase A and human disease. Trends Endocrinol Metab. 2002;13:50-52.
14. Stratakis CA, Miller WR, Severin E, Chin KV, Bertherat J. Amieux PS, Eng C, Kammer GM. Dumont JE, Tortora G, Beaven MA, Puck TT, Jan De Beur SM, Weistein LS, Cho-Chung YS. Protein-kinase A and human disease: the core of cAMP-dependent signaling in health and disease. Horm Metab Res. 2002;34:169-175.

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# REGULATION OF SKELETAL GROWTH

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Robert Mitchum, *Technical Training Fellow*
Ellen Leschek, M.D., *Special Volunteer*

We investigate the cellular and molecular mechanisms governing bone growth and development. One of our goals is to improve medical treatment of growth disorders and childhood metabolic bone diseases. In addition, given that the cellular processes underlying bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are also essential for development in other tissues, we seek to uncover general principles of developmental biology.

## Developmental Regulation of Growth Plate Chondrogenesis

*Baron, Barnes, Nilsson, Gafni, Flor, Nwosu, Mitchum*

Longitudinal bone growth occurs at the growth plate, a thin layer of cartilage that lies near the ends of long bones and vertebrae. The growth plate consists of three principal layers: the resting zone, the proliferative zone, and the hypertrophic zone. We have demonstrated that the resting zone contains stem-like cells that are capable of generating new clones of proliferative chondrocytes. We have also shown that the resting zone directs the spatial orientation of the proliferative clones, causing them to form columns parallel to the long axis of the bone. These proliferative cells undergo clonal expansion followed by cellular hypertrophy. The hypertrophic cartilage is then remodeled into bone tissue. The net effect is that new bone tissue is progressively created at the bottom of the growth plate, resulting in bone elongation.

Overall body proportions are determined by the size of the skeleton, which, in turn, is determined by the rate and duration of longitudinal bone growth. The rate falls progressively with age. In humans, fetal growth exceeds 100 centimeters per year. By birth, the growth rate has decreased to 50 centimeters per year, and by mid-childhood, five centimeters per year. A similar progressive decline in bone growth occurs in other mammals. The differences among mammals in final skeletal size (e.g., between a mouse and an elephant) are determined largely by the rapidity of the decline. The decline in growth rate with increasing age is due primarily to a decrease in the rate of growth plate chondrocyte proliferation. Our *in vivo* studies suggest that the decrease in chondrocyte proliferation occurs because stem-like cells of the growth plate have a finite proliferative capacity, which is gradually exhausted. Early in life, when the stem-like cells have a great proliferative capacity, the growth plate chondrocytes replicate rapidly, causing rapid longitudinal bone growth. Later in life, when the stem-like cells have expended much of their proliferative capacity, the derivative clones of chondrocytes replicate more slowly, resulting in slower longitudinal bone growth. The cellular and molecular mechanisms that limit proliferation of growth plate chondrocytes are currently under investigation.

Growth-inhibiting conditions, such as glucocorticoid excess, slow the proliferation of growth plate chondrocytes and thus conserve the proliferative capacity of these cells. If the growth-inhibiting condition resolves, the chondrocytes would therefore retain a greater proliferative capacity than normal and thus would proliferate more rapidly and for a longer period of time. Therefore, the conservation of proliferative capacity provides an explanation for catch-up growth, which has been observed in both humans and other mammals. We have demonstrated this mechanistic explanation *in vivo*.

Eventually, in a process known as epiphyseal fusion, growth ceases and the growth plate is replaced by bone. Our findings suggest that epiphyseal fusion is triggered when the proliferative capacity of the growth plate chondrocytes is finally exhausted. We have found evidence that estrogen accelerates the proliferative exhaustion of the growth plate chondrocytes. As a result, estrogen leads to early termination of linear growth and early epiphyseal fusion. Consistent with this hypothesis, we have found that both alpha and beta estrogen receptors are expressed in growth plate chondrocytes throughout postnatal development in rats and rabbits.

We are currently conducting a clinical study to determine whether estrogen accelerates proliferative exhaustion in human growth plate chondrocytes. We are analyzing growth data from girls with precocious puberty treated with a gonadotropin-releasing hormone analog (GnRHa). In these girls, the precocious puberty caused the growth plates to be exposed to high levels of estrogen. Our animal studies suggest that such estrogen exposure would cause accelerated proliferative exhaustion of growth plate chondrocytes. Treatment with GnRHa causes normalization of estrogen levels. However, if the previous estrogen exposure exhausts the proliferative capacity of the growth plate chondrocytes, we would expect the growth rate to be low during the GnRHa treatment. Preliminary analysis of the growth data is consistent with this prediction. Furthermore, the severity of the growth impairment appears to be correlated with the severity of the estrogen exposure before treatment. Thus, the preliminary analysis suggests that estrogen does accelerate proliferative exhaustion of growth plate chondrocytes in humans as it does in other mammals.

## Developmental Regulation of Skeletal Strength

*Baron, Barnes, Gafni*

The process of bone growth not only determines body size but also affects the structural integrity of the skeleton. Thus, understanding skeletal growth may provide insights into the origins of osteoporosis. However, most basic and clinical studies of bone formation and osteoporosis are performed in adult individuals, focusing on the process of bone remodeling. Far less attention has been paid to the juvenile skeleton, in which bone is not only remodeled but also created through growth.

It is often assumed that decreased bone mineral acquisition during childhood will cause a permanent decrease in bone mineral density, which will increase the risk of fractures in late adulthood. To the contrary, we found evidence that bone mineral acquisition early in life has little or no effect on adult bone mass because many areas of the juvenile skeleton are replaced *in toto* through skeletal growth. In the growing long bone, new trabeculae are formed adjacent to the growth plate. Older trabeculae are resorbed as they approach the medullary cavity. Therefore, trabeculae formed early in childhood are completely replaced by new trabeculae later in childhood. Similarly, cortical bone is resorbed at its endosteal (interior) surface and extended at its periosteal (exterior) surface. As a result, cortical bone formed in early childhood is completely replaced over time. We have demonstrated these phenomena *in vivo* and have shown that they can cause recovery even from severe osteoporosis in a growing animal. Thus, our data suggest that bone mineral acquisition in early life has little effect on adult bone density. If this concept generalizes to humans, then interventions to maximize peak bone mass would be more effective if directed at adolescents rather than at young children.

### Clinical Studies

*Gafni, Leschek, Baron*

We are conducting a clinical trial of alendronate for the treatment of idiopathic juvenile osteoporosis. Children with idiopathic juvenile osteoporosis are randomly assigned to receive either alendronate or placebo. Alendronate, a bisphosphonate that can be administered orally, inhibits bone resorption. It has proven effective in the treatment of postmenopausal osteoporosis and glucocorticoid-induced osteoporosis in adults. The purpose of our study is to determine whether the medication will provide a safe and effective treatment for childhood osteoporosis.

We are also completing a randomized double-blind placebo-controlled trial of growth hormone therapy in children with non-growth-hormone–deficient extreme short stature. Currently, thousands of children with extreme non-growth-hormone–deficient short stature receive growth hormone therapy despite the absence of conclusive data regarding long-term efficacy and safety. Non-randomized long-term studies have yielded conflicting results as to whether growth hormone therapy increases the adult height of children without growth hormone deficiency. This public health issue was foreseen in 1983; the Conference on Uses and Possible Abuses of Biosynthetic Human Growth Hormone, convened by the NICHD, concluded that "there is an urgent need for therapeutic trials to determine the effect of growth hormone in short children who do not have a growth hormone deficiency." Similarly, in 1987, the FDA Endocrinologic and Metabolic Drugs Advisory Committee called for well-controlled studies on the long-term safety and efficacy of growth hormone for children who are not growth hormone–deficient. In response, NICHD initiated the current study. The final data are currently under analysis.

## SELECTED PUBLICATIONS

1. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, Bacher JD, Baron J. The role of the resting zone in growth plate chondrogenesis. Endocrinology. 2002;143:1851-1857.
2. De Luca F, Barnes KM, Uyeda JA, De-Levi S, Abad V, Palese T, Mericq MV, Baron J. Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. Endocrinology. 2001;142:430-436.
3. De Luca F, Uyeda JA, Mericq V, Mancilla EE, Yanovski JA, Barnes KM, Zile MH, Baron J. Retinoic acid is a potent regulator of growth plate chondrogenesis. Endocrinology. 2000;141:346-353.
4. Gafni RI, Baron J. Catch-up growth: possible mechanisms. Pediatr Nephrol. 2000;14:616-619.
5. Gafni RI, McCarthy EF, Hatcher T, Meyers JL, Inoue N, Reddy C, Weise M, Barnes KM, Abad V, Baron J. Recovery from osteoporosis through skeletal growth: early bone mass acquisition has little effect on adult bone density. FASEB J. 16:736-738.
6. Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De-Levi S, Baron J. Catch-up growth is associated with delayed senescence of the growth plate in rabbits. Pediatr Res. 2001;50:618-623.
7. Leschek EW, Troendle JF, Yanovski JA, Rose SR, Bernstein DB, Cutler GB, Baron J. Effect of growth hormone treatment on testicular function, puberty, and adrenarche in boys in non-growth hormone-deficient short stature: a randomized, double-blind, placebo-controlled trial. J Pediatr. 2001;138:406-410.
8. Mericq MV, Baron J. $Ca^{2+}$-sensing receptor abnormalities. In: Chrousos GP, Olefsky JM, Samols E, eds. Hormone resistance and hypersensitivity states. Philadelphia: Lipincott-Raven, 2002;289-300.
9. Mericq MV, Uyeda JA, De Luca F, Baron J. Regulation of fetal rat bone growth by C-type natriuretic peptide and cGMP. Pediatr Res. 2000;47:1-5.
10. Nilsson O, Abad V, Chrysis D, Ritzen EM, Savendahl L, Baron J. Estrogen receptor-alpha and beta are expressed throughout postnatal development in the rat and rabbit growth plate. J Endocrinol. 2002;173:407-414.
11. Rose SR, Baron J, Bernstein D, Yanovski J, Troendle JF, Leschek E, Chipman JJ, Cutler GB. Suppression and recovery of GH secretion after GH injection in non-GH-deficient children. J Pediatr Endocrinol Metab. 2000;13:281-288.
12. Weise M, De-Levi S, Barnes KM, Gafni RI, Abad V, Baron J. Effects of estrogen on growth plate senescence and epiphyseal fusion. Proc Natl Acad Sci USA. 2001;98:6871-6876.

# NEUROENDOCRINOLOGY OF STRESS

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Our goal is to understand the neuroendocrine mechanisms underlying the stress response, with emphasis on the regulation of the hypothalamic pituitary adrenal (HPA) axis. The ability of the organism to adapt to acute and chronic stress situations is determined by genetic constitution and previous experiences. Studies in our laboratory have shown that exposure to a repeated somatosensory stress causes hyper-responsiveness of the HPA axis to a novel stress. Given that hyperactivity of the HPA axis has been implicated in the pathogenesis of several psychiatric and metabolic disorders, self-limitation of the stress response is critical for avoiding deleterious effects of glucocorticoid excess. Our laboratory studies the mechanisms by which the expression of the hypothalamic hormones, corticotropin releasing hormone (CRH) and vasopressin (VP) and their pituitary receptors, are regulated under different stress situations as well as the consequences of such regulation on adrenocorticotropic hormone (ACTH) secretion and adrenal steroidogenesis

## Regulation of Hypothalamic CRH and VP Expression

*Kalintchenko, Nikodemova, Shepard*

Studies in the laboratory have been pivotal in understanding the interaction between CRH and VP in the regulation of pituitary ACTH and the regulation of the expression of these peptides in the paraventricular nucleus (PVN) during stress and other alterations of the HPA axis. Previous studies showed that CRH and VP co-expressed in the same parvocellular neuron of the PVN are differentially regulated during stress or exposure to glucocorticoids, with VP becoming the predominant regulator during chronic stress. During the past year, our studies have focused on the mechanisms by which prolonged stress results in lowered CRH transcription but sustained increases in VP in the parvocellular neuron. Characterization of a recently produced fetal rat hypothalamic cell line revealed expression of endogenous CRH, VP, and glucocorticoid receptors, suggesting a parvocellular neuron phenotype. Transfection of CRH or VP promoter reporter gene constructs into this cell line revealed that activation of VP promoter required much higher levels of cAMP than activation of the CRH promoter. High levels of cAMP also stimulate the expression of inhibitory isoforms of cAMP-responsive element modulator (CREM). We showed that inhibitory isoforms of CREM cause marked repression of cAMP-stimulated CRH promoter but had no effect on cAMP-stimulated VP promoter activity. This diverse response of the CRH and VP promoters to cAMP-dependent signaling could contribute to the differential regulation of both genes during stress.

## Self-Limitation of Stress Responses

*Nikodemova, Shepard, Aguilera*

We are conducting *in vivo* and *in vitro* studies to determine the mechanisms responsible for termination of the stress response. A recognized mediator of negative feedback during the HPA axis response is the effect of increased circulating glucocorticoids in the brain and pituitary. However, stress causes refractoriness to the inhibitory effect of glucocorticoids, leading to ineffectiveness of the feedback mechanism. Studies are under way to elucidate molecular mechanisms modulating the effectiveness of glucocorticoid feedback and the role of neurotransmitters such as GABA and to elaborate on autoregulatory mechanisms in hypothalamic neurons in the self-limitation of HPA axis responses to stress.

While CRH is essential for stress response, we have shown that the increases in CRH transcription during stress are transient even if the stimulus is sustained. Research in the laboratory has focused on determining the mechanisms responsible for turning on and off CRH transcription during stress. Evidence suggests that cAMP-dependent signaling plays an important role in the activation of CRH transcription in non-hypothalamic cells. During the last year, we used the rat hypothalamic cell line 4B to investigate tissue specificity of cAMP-dependent regulation of a 498 bp CRH promoter fragment. The data showed that minimal elevations in intracellular cAMP are sufficient to activate CRH transcription fully irrespective of the cell line. Rapid degradation of cAMP by phosphodiesterases may contribute to the turn-off of the CRH transcriptional response to cAMP. Elevations in intracellular cAMP also appear to be involved in self-limiting CRH transcription during stress by inducing the expression of inhibitory CREMs. *In vivo*, there is an increase in expression of the CREM isoform, inducible cAMP early repressor (ICER), in the PVN corresponding to the decreasing phase of CRH transcription. In addition, co-transfection of CRH promoter–luciferase constructs and ICER in a hypothalamic cell line inhibits cAMP-stimulated CRH promoter activity *in vitro*, suggesting that the ICER mediates a cellular feedback mechanism to limit CRH transcriptional responses during prolonged stress.

### Neuroendocrine Immune Interactions

*Grinevich,* Lightman, Tilders, Aguilera*

Single exposure to the proinflammatory cytokine interleukin-1 (IL-1) induces sensitization of the ACTH and corticosterone responses to stressors weeks after HPA axis sensitization. Studies were conducted to determine whether sensitization of the HPA responses to a novel stressor (novelty) involves autoexcitatory feedback mediated by CRH and VP receptors in the hypothalamic PVN. Single administration of IL-1 induced sensitization of the HPA to novelty from three to 22 days later and biphasic increases in CRH and CRH-R1 mRNAs in the PVN: an early peak within 24h, followed by a delayed (more than 7 days) increase that peaked after 22 days. Hypothalamic V1a and V1b mRNA levels were unaffected. In contrast, in the pituitary gland, there was an early decrease in CRH-R1 mRNA and V1b receptor mRNA, which returned to control levels from 24h onwards. In contrast to the prolonged activation of CRH and CRH-R1 mRNA expression in the PVN, there were no changes in the expression of the ACTH precursor molecule, POMC, V1b receptor, or CRH R1 mRNAs in the pituitary gland. These studies indicate that long-lasting hypothalamic adaptations that result in enhanced central CRH signaling contribute to the long-term sensitization of the HPA axis to transient exposure to immune events.

One particularly exciting finding was that chronic inflammatory stress in a rat model of autoimmune arthritis causes hypersensitivity of the HPA axis and the immune system to a novel immune challenge. Rats with adjuvant-induced arthritis show hyperactivity of the HPA axis accompanied by paradoxical decreases in CRH expression and increases in VP expression in parvocellular neurons of the PVN. In spite of blunted HPA axis responses to psychosensory stressors, the rats showed normal or exacerbated responses to the novel immune challenge of lipopolysaccharide (LPS) injection compared with control rats. Arthritic rats also showed much higher plasma IL-1 and IL6 levels and IL-1 and IL-6 mRNA in the brain than control rats after LPS injection. The data reveal an increased peripheral and central immunological response to LPS challenge during the chronic inflammatory process of arthritis. The fact that chronic inflammatory disorders can influence the responses to a novel immune challenge and enhance the induction of interleukins in the brain and periphery may be relevant for the pathogenesis of cytokine-mediated chronic degenerative disorders.

### Regulation of Pituitary CRH and V1b VP Receptors

*Rabadan-Diehl, Volpi*

Regulation of the number of CRH and VP receptors in the pituitary plays an important role in the control of HPA axis activity. Studies in our laboratory have shown that CRH receptor content in the pituitary does not depend on the levels of CRHR1 mRNA, indicating that regulation of the number of functional receptors occurs at post-transcriptional sites. During the last year, the laboratory has focused on the regulation of the VP receptor V1b, which is the major subtype in the pituitary corticotroph. Our laboratory demonstrated that increased pituitary corticotroph responsiveness during chronic stress is associated with VP receptor up-regulation. Studies on the transcriptional regulation of the V1b VP receptor have identified a region in the proximal promoter containing a large GAGA repeat, which is essential for positive regulation of the V1b receptor promoter and binds to a protein complex found in pituitary nuclear extracts. The protein complex appears to bind to DNA as a dimer of 70 kDa subunits. Transfection of a GAGA binding protein described in *Drosophila* markedly enhances V1b receptor promoter activity as well as the expression of endogenous V1b receptor in a hypothalamic cell line. In addition, stress increases the GAGA binding protein activity of pituitary nuclear extracts, suggesting that a GAGA binding protein plays a role in the physiological regulation of V1b receptor transcription. Studies of the full characterization of the GAGA binding protein complex and the role of other transcription factors on the regulation of the V1b receptor gene are ongoing.

Previous studies conducted in this laboratory indicated a lack of correlation between V1b receptor mRNA and V1b receptor binding, suggesting that regulation at post-transcriptional sites plays an important role in determining the number of VP receptors in the pituitary. In addition, studies during the past year showed that the 5' untranslated region (5'UTR) of the V1b receptor mRNA plays a significant role in controlling translation of the mRNA to receptor protein. The presence of upstream open reading frames in the 5'UTR may play a role in maintaining low translational activity in basal conditions. On the other hand, studies during the past year have identified an internal ribosome entry site (IRES) in the 5'UTR. IRES activity can be stimulated by activation of protein kinase C and PI3 kinase–dependent pathways. These data provide mechanisms by which the 5'UTR of the V1b mRNA can determine negative and positive regulation for translation of V1b receptor mRNA according to the physiological requirements.

## SELECTED PUBLICATIONS

1. Aguilera G. Signal transduction: receptors. In: Izzo JL, Black HR. eds. Hypertension primer. American Heart Association. 2002;in press.
2. Aguilera G, Lightman SL, Ma X-M. Transcriptional and post-transcriptional regulation of corticotropin releasing hormone and vasopressin expression by stress and glucocorticoids. In: McCarty R, Aguilera G, Kvetnansky R. eds. Stress: neural, endocrine and molecular studies. New York: Taylor and Francis, 2002;91-97.
3. Aguilera G. Rabadan-Diehl C, Kiss A, Ochedalski T. Vasoactive hormones and regulation of the hypothalamic-pituitary-adrenal axis. In: Nagatsu T, Nabeshima T, eds. Catecholamine research: from molecular insights to clinical medicine. Kluwer: Academic/Plenum. 2002:281-284.
4. Aguilera G, Rabadan-Diehl C, Nikodemova M. Regulation of pituitary corticotropin releasing hormone receptors. Peptides. 2001;22:769-774.
5. Arima H. House SB, Gainer H, Aguilera G. Direct stimulation of Arginine Vasopressin gene transcription by cyclic adenosine monophosphate in parvocellular neurons of the paraventricular hypothalamic nucleus. Endocrinology. 2001;142:5027-5030.

6. Arima H, House SB, Gainer H, Aguilera G. Neuronal activity and MAP kinase dependent pathways are required for the diurnal rhythm of vasopressin gene transcription in the suprachiasmatic nucleus in vitro. Endocrinology. 2002;143:4165-4171.
7. DaCosta A, Ma XM, Ingram C, Lightman SL, Aguilera G. Hypothalamic and amygdaloid corticotropin releasing hormone and CRH receptor mRNA expression in the stress-hyporresponsive pregnant and lactating rat. Mol Brain Res. 2001;91:119-130.
8. Grinevich V, Harbuz M, Ma XM, Jessop D, Tilders FJH, Lightman SL, Aguilera G. Hypothalamic-pituitary-adrenal axis and immune responses to endotoxin in rats with adjuvant-induced arthritis. Exp Neurol.;in press.
9. Grinevich V, Ma XM, Herman JP, Jezova D, Akmayev I, Aguilera G. Effect of repeated lypopolysaccharide administration on tissue cytokine expression and hypothalamic pituitary adrenal axis activity in rats. J Neuroendocrinol. 2001;13:711-723.
10. Grinevich V, Ma XM, Verbalis J, Aguilera G. Hypothalamic pituitary adrenal axis and hypothalamic neurohypophyseal responses to restraint or immune challenge in water deprived rats. Exp Neurol. 2001;171:329-341.
11. Nikodemova M, Rabadan-Diehl C, Aguilera G. Multiple levels of regulation controlling type-1 corticotropin-releasing hormone receptors levels in the pituitary. Arch Physiol Biochem. 2002;110:123-128.
12. Rabadan-Diehl C, Aguilera G. Pituitary CRH and VP receptors and their role in corticotroph responsiveness during stress. In: McCarty R, Aguilera G, Kvetnansky R, eds. Stress: neural, endocrine and molecular studies. New York: Taylor and Francis, 2002;227-231.
13. Volpi S, Rabadan-Diehl C, Aguilera G. Transcriptional regulation of the pituitary vasopressin V1b receptor involves a GAGA binding protein. J Biol Chem. 2002;277:27829-27838.
14. Xu G-H, Rabadan-Diehl C, Nikodemova M, Wynn P, Spiess J, Aguilera G. Inhibition of corticotropin releasing hormone type-1 receptor translation by an upstream AUG triplet in the 5' unstranslated region in the mRNA. Mol Pharmacol. 2001;59:485-492.

## COLLABORATORS

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# GROWTH AND OBESITY

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The prevalence of overweight and obesity in children and adults has doubled during the past 20 years. The alarming rise in body weight has likely occurred because the current environment affords easy access to calorie-dense foods and requires less voluntary energy expenditure. However, such an environment leads to obesity only in those individuals whose body weight regulatory systems are not able to control body adiposity with sufficient precision in our high-calorie/low-activity environment, which suggests that some subgroups in the United States have a uniquely high susceptibility to weight gain under the prevailing environmental conditions. Our research is directed at increasing our understanding of the metabolic and behavioral factors involved in determining body weight regulation and body composition during childhood, with a special emphasis on minority populations. Our ongoing research program prospectively evaluates risk factors for the development of obesity and its complications in children, studies the effects of anorectic medications on body weight and obesity-related comorbid conditions in children, and seeks the genetic and environmental factors important for the markedly greater incidence of obesity and its comorbid conditions in some U.S. minority populations. A second research direction examines the pathophysiology of HIV-associated lipodystrophy in children and adults.

### Molecular Studies of Childhood Body Weight Regulation

*Feng, Adler-Wailes, Elberg, Carr, Liu, Yanovski J; in collaboration with Warden*

Using classical association studies, we are studying polymorphisms in genes involved in the leptin signalling pathway and thus are attempting to identify gene variants with an impact on body composition that differ in frequency between African American and Caucasian children. Genes currently under study include proopiomelanocortin (POMC), POMC processing enzymes, the melanocortin receptors 3, 4, and 5, and neuropeptide Y and its receptors. In addition, we have studied genes important for energy expenditure, such as the mitochondrial uncoupling proteins, and genes potentially involved in cortisol metabolism that may influence intra-abdominal adipose tissue, such as 11-beta-hydroxysteroid dehydrogenase. We have found that a 45 base-pair insertion polymorphism in the eighth exon of UCP-2 is associated with excess body weight in African American, Asian, and Caucasian children. We have also identified novel polymorphisms in the POMC sequence that are more prevalent in African American than Caucasian children but do not appear to play a role in the greater body adiposity of African American children. A polymorphism in the melanocortin 3 receptor that is associated with adiposity in a large cohort of children appears to have functional significance for signal transduction. Ongoing studies attempt to understand the mechanisms by which these sequences alterations may affect body weight.

### Physiology, Metabolism, and Psychology of Childhood Body Weight Regulation

*Uwaifo, McDuffie, Bonat, Parikh, Elberg, Fallon, Nguyen, Morgan, Tanofsky-Kraff, Yanovski J; in collaboration with Young-Hyman, Schoeller, Warden, Yanovski S, Keil*

Given that the amount of visceral fat in Caucasians is highly associated with the complications of obesity, we have studied the distribution of adipose tissue in African American and Caucasian children along with methods for assessing children's insulin resistance and body composition. We have found less visceral abdominal adipose tissue in non-obese and obese African American children than in Caucasian children but considerably greater insulin resistance in African American children. In recent studies, the hyperglycemic clamp procedure in children was highly correlated with the more technically demanding euglycemic, hyperinsulinemic clamp study and proved suitable for assessing children's insulin sensitivity. African American children were then found to have greater insulin secretion, a greater prevalence of acanthosis nigricans, and less insulin sensitivity than Caucasian children of similar body composition. The results imply that the relationship between visceral fat and the complications of obesity may be different in African Americans and Caucasians. The susceptibility to weight gain in African American children may also result from differences in metabolic efficiency. We have also found that resting energy expenditure is approximately 90 kcal/d less in African American than in Caucasian normal-weight and overweight boys and girls. Our studies suggest that the differences are not explained by differences in the hormone leptin. We have also studied the effects of adiposity on skeletal maturation in African American and Caucasian children, finding that a substantial portion of the advancement of sexual and skeletal maturation found in African American children may be explained by their greater adiposity.

In two ongoing protocols (83-CH-0169 and 96-CH-0101) designed to determine the factors that are most important for development of the complications of obesity in African American and Caucasian youth, we are studying normal-weight African American and Caucasian children and adolescents, already obese African American and Caucasian children, and the non-obese African American and Caucasian children of obese parents. We are examining body composition, metabolic rate, insulin sensitivity, glucose disposal, and genetic factors believed to regulate metabolic rate such as the uncoupling proteins, leptin and its receptor, and the beta-3 adrenergic receptor. We are also studying psychological and behavioral factors, such as propensity to engage in binge eating behavior. We have recently found that children who report binge eating episodes during childhood have greater adiposity than those not reporting such episodes. Children are being studied longitudinally into adulthood. We hypothesize that differences in various factors will predict the development of obesity in the populations studied and may be of great importance in developing rational approaches for the prevention and treatment of obesity in the diverse U.S. population.

### Treatment of Children and Adolescents with Comorbid Conditions Associated with Obesity

*Uwaifo, McDuffie, Bonat, Parikh, Fallon, Elberg, Chin, Tanofsky-Kraff, Yanovski J; in collaboration with Drinkard, Sebring, Salaita, Riggs, Young-Hyman, Calis, Reynolds, Krakoff, Rolls*

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in childhood is urgently needed. In two ongoing clinical protocols, we are studying novel approaches for controlling body weight in children. We have completed a pilot study demonstrating that severely overweight adolescents can lose weight when enrolled in a comprehensive weight management program that includes the novel gastrointestinal lipase inhibitor orlistat as an adjunct to a behavioral modification program. We have also found evidence that one mechanism through which orlistat may affect body weight is by changing the hedonic value of dietary fat. A placebo-controlled randomized trial will determine whether the use of orlistat improves the weight loss of African American and Caucasian children and adolescents with obesity-related comorbidities. A second study examines the mechanism by which another novel weight loss agent, metformin, may affect the body weight of younger children with hyperinsulinemia.

### Etiology of HIV-Associated Lipodystrophy

*Adler-Wailes, Feng, Yanovski J*

We are actively engaged in understanding the syndrome of HIV-associated lipodystrophy. Since it was initially recognized in 1982, acquired immunodeficiency syndrome (AIDS) has been a leading cause of death among children and young adults in the U.S. for much of the last decade. Following the introduction of protease inhibitors as part of "highly active antiretroviral therapy" (HAART) for the treatment of HIV-infected patients, AIDS-related morbidity and mortality decreased dramatically. However, HIV-infected children and adults, particularly those treated with protease inhibitors, often develop a lipodystrophy that includes significant changes in body shape, with fat loss in the face, arms, and legs and fat gain in the trunk. This lipodystrophy is often accompanied by hypertriglyceridemia, hypercholesterolemia, and hyperinsulinemia. Understanding the pathophysiology of lipodystrophy is therefore important for the long-term therapy of HIV infection. In addition, given that certain types of body habitus are associated with increased risks of cardiovascular disease in non–HIV-infected individuals, an enhanced understanding of the control of adipocyte physiology and fat distribution may have additional significance for the broader population. We have studied the etiology of HIV-associated lipodystrophy in clinical populations; we reported an increase in visceral adipose tissue in HIV-infected adults, found marked abnormalities in both insulin sensitivity and control of cholesterol and triglyceride metabolism, and examined the metabolic consequences of interrupting HAART. Subsequent studies have ruled out alterations in the hypothalamic-pituitary-adrenal axis as causes of this syndrome. In our ongoing studies, we are attempting to elucidate the alterations in adipocyte function that arise as a result of exposure to the components of HAART. Preliminary analyses suggest that the protease inhibitor ritonavir significantly affects hormone-stimulated lipolysis as well as transcription of numerous adipocyte-specific and non–adipocyte-specific genes.

### Environmental Factors Affecting Adult Weight Gain

*Parikh, Nguyen, Sovik, Chin, Yanovski J; in collaboration with Yanovski S*

We are also interested in assessing the impact of the environment on body weight gain. To determine the role of seasonal variation in weight change, we prospectively studied 200 U.S. adults, following them longitudinally. We found that the only time period during which weight changes significantly is during the fall-winter holiday season between Thanksgiving and New Year's Day. Such studies have implications for individuals attempting to control their weight. We are also interested in the role dietary calcium plays in determining body weight change. A new protocol attempts to examine the impact of dietary calcium supplementation on body weight in a randomized, controlled trial of 340 adults.

## SELECTED PUBLICATIONS

1. Drinkard B, McDuffie J, McCann S, Uwaifo G, Nicholson J, Yanovski JA. Walk/run performance in overweight adolescents. Phys Ther. 2001;81:1889-1896.
2. McDuffie JR, Calis KA, Booth S, Uwaifo GI, Yanovski JA. Effects of orlistat on fat-soluble vitamins in obese adolescents. Pharmacotherapy. 2002;22:814-822.
3. McDuffie JR, Calis KA. Uwaifo GI, Sebring NS, Fallon EM, Hubbard VS, Yanovski JA. Three-month tolerability of orlistat in adolescents with obesity-related comorbid conditions. Obese Res.2002;10:642-650.

4. Morgan CM, Yanovski SZ, Nguyen TT, McDuffie J, Sebring N, Jorge M, Keil M, Yanovski JA. Loss of control over eating, adiposity, and psychopathology in overweight children. Int J Eat Disord. 2002;31:430-441.
5. Nguyen TT, Keil MF, Russell DL, Pathomvanich A, Uwaifo GI, Sebring NG, Reynolds JC, Yanovski JA. Relation of acanthosis nigricans to hyperinsulinemia in overweight African American and white children. J Pediatr. 2001;138:474-480.
6. Russell DL, Keil MF, Bonat SH, Uwaifo GI, Nicholson JC, McDuffie JR, Hill SC, Yanovski JA. The relationship between skeletal maturation and adiposity in African American and Caucasian children. J Pediatr. 2001;139:844-848.
7. Uwaifo GI, Elberg J, Yanovski JA. Impaired glucose tolerance in obese children and adolescents. N Engl J Med. 2002;347:290-292.
8. Uwaifo GI, Nguyen TT, Russell DL, Keil MF, Nicholson JC, Bonat SH, McDuffie JR, Yanovski JA. Differences in insulin secretion and sensitivity of Caucasian and African American prepubertal children. J Pediatr. 2002;140:673-680.
9. Yanovski JA. Intensive therapies for pediatric obesity. Pediatr Clin North Am. 2001;48:1041-1053.
10. Yanovski JA, Diament AL, Sovik KN, Nguyen TT, Li H, Sebring N, Warden CH. Associations between Uncoupling Protein 2, body composition, and resting energy expenditure in lean and obese African American, white, and Asian children. Am J Clin Nutr. 2000;71:1405-1420.
11. Yanovski JA, Yanovski SZ, Sovik KN, Nguyen TT, O'Neil PM, Sebring N. A prospective study of holiday weight gain. N Engl J Med. 2000;342:861-867.
12. Yanovski SZ, Yanovski JA. Pharmacotherapy for obesity. N Engl J Med. 2001;346:2092-2093.

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# THE MENSTRUAL CYCLE AND HUMAN REPRODUCTION

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Using autoimmune premature ovarian failure as a model condition, we investigate genetic, immunological, and molecular aspects of menstrual cycle disorders and disorders of human reproduction in order to develop diagnostic tools and therapeutic approaches for such disorders. Premature ovarian failure (POF) causes young women to develop amenorrhea and infertility before age 40 and was once considered an irreversible condition similar to normal menopause. We now know that 50 percent of POF patients have follicles remaining in the ovary, even though the follicles fail to function normally. In 90 percent of cases, the mechanism of the ovarian insufficiency remains a mystery. Work in a mouse model of autoimmune POF, neonatal thymectomy of B6A mice, which induces autoimmune oophoritis and ovarian failure similar to human autoimmune ovarian failure, led us to discover Mater, a novel oocyte protein that serves as a major antigen in ovarian autoimmunity. Using transgenic technology, we also demonstrated that Mater is essential for normal female fertility. In addition, we have been investigating clinical POF by recruiting patients to research protocols that are designed to gain insight into the mechanisms of ovarian follicle dysfunction and to develop appropriate treatments for patients with 46, XX spontaneous POF.

## *MATER*, a Human Gene Critical to Female Fertility

*Tong, Nelson*

During the past year, we defined the human homolog of the mouse *Mater,* a gene critical to female fertility. We previously reported that *Mater* is a maternal effect gene required for early embryonic development in mice. We cloned human MATER cDNA with PCR techniques, used hybridization and immunodetection to determine, respectively, the mRNA and protein, and relied on bioinformatics software to analyze the cDNA and protein sequences. We found that the human MATER gene spans an approximately 63 kbp DNA at chromosome 19 and is composed of 15 exons and 14 introns. Expression of its mRNA (about 4.2 kb) is restricted to the oocyte. Human MATER cDNA (3885 nt) shows an open reading frame (3600 nt) encoding a polypeptide chain composed of 1,200 residues with a predicted molecular mass of 134,236 Da. We detected MATER protein in human oocytes. The human and mouse cDNA share 67 percent homology while their deduced polypeptide chains exhibit 53 percent sequence identity. Further, their protein structures have a number of similar features. We have concluded that the human *MATER* and mouse *Mater* genes and their encoded proteins are conserved. Characterization of the human *MATER* and its protein provides a basis for us to investigate their clinical implications in autoimmune premature ovarian failure and infertility in women.

We have generated a mouse line lacking *Mater* and are investigating the *Mater* mouse as a model for human idiopathic infertility. Some couples experience infertility despite the fact that no abnormalities can be detected in the man or the woman. A portion of these cases might be explained by poor egg quality related to genetic or functional deficiencies in heretofore unidentified human maternal effect genes. Maternal effect genes produce mRNA or proteins that accumulate in the egg during oogenesis, and these maternal products control the developmental program until the embryonic genome is activated. The factors governing the transition from the maternal to the embryonic genome are unknown. In mice, embryonic transcription is first detected in the late one-cell zygote stage and is required for development beyond the two-cell stage. Embryos from *Mater* female mice have a block in development at the two-cell stage.

### Needs of Young Women with 46,XX Spontaneous Premature Ovarian Failure

*Pastor, Groff, Vanderhoof, Nelson; in collaboration with Al-Zubaidi, Calis*

The infertility associated with premature ovarian failure has been the primary focus of our research. An in-depth understanding of the mechanisms of follicle dysfunction in young women with 46,XX spontaneous premature ovarian failure might lead to treatments that could restore fertility. However, young women with the condition have other needs that must be met by their health care providers. We are conducting clinical research to define these other needs more fully and to determine how clinicians might best respond to them.

During the past year, we investigated the experiences of young women with spontaneous premature ovarian failure with regard to their initial presenting symptom, promptness of diagnosis, and patient education. We asked 50 patients previously diagnosed with spontaneous premature ovarian failure to participate in a structured interview survey. Disturbance in menstrual pattern was the most common initial symptom. Over half of the women who presented with this complaint reported visiting a clinician's office three or more times before laboratory testing was performed to determine the diagnosis. For 25 percent of the women, more than five years elapsed before the diagnosis of premature ovarian failure was established. A majority of the women were dissatisfied with the manner in which they had been informed of the diagnosis and the amount of information they had received about their condition. Our findings demonstrate that women with spontaneous premature ovarian failure perceive a need for more aggressive evaluation of secondary amenorrhea and oligomenorrhea. Loss of menstrual regularity can be a sign of ovarian insufficiency, and the associated estrogen deficiency is a well-established risk factor for osteoporosis. Indeed, in an earlier study we found that two-thirds of young women with spontaneous premature ovarian failure had osteopenia of the femoral neck.

### Autoimmune Adrenal Insufficiency

*Vanderhoof, Nelson; in collaboration with Bakalov, Bondy*

It is well established that patients with spontaneous premature ovarian failure are at increased risk of developing autoimmune adrenal insufficiency, a potentially fatal disorder. Adrenal insufficiency has an insidious onset, and patients with the condition often experience a delay in diagnosis. There has been ongoing controversy regarding the best strategy to detect adrenal insufficiency at an early stage in these young women. During the past year, we completed studies testing the hypothesis that anti-steroid cell antibodies detected by indirect immunofluorescence may be an effective screening test. We comprehensively evaluated the adrenal axis of 123 women with spontaneous premature ovarian failure. We uncovered a new diagnosis of asymptomatic adrenal insufficiency in four women (3.2 percent). All four tested positive for adrenal antibodies. A positive adrenal antibody test was highly associated with adrenal insufficiency while a negative test was associated with normal adrenal function in all cases. The presence of positive adrenal antibodies increased the pretest probability of adrenal insufficiency from 3.2 to 67 percent. As a screening method, the cortisol response during a standard adrenocorticotrophic hormone (ACTH) stimulation test gave two false positive results.

Our findings demonstrate that measuring adrenal antibodies is an effective screening method with which to detect asymptomatic autoimmune adrenal insufficiency in young women with spontaneous premature ovarian failure. The standard ACTH stimulation test should be reserved to confirm adrenal insufficiency in women who test positive for adrenal antibodies or in those with signs and symptoms of adrenal insufficiency.

### Mechanisms of Follicle Dysfunction in 46,XX Spontaneous Premature Ovarian Failure

*Anasti, Shibanuma, Tong, Udofa, Emeche, Nelson; in collaboration with Bakalov*

Autoimmune lymphocytic oophoritis is a clearly established mechanism of follicle dysfunction in some patients with 46,XX spontaneous premature ovarian failure. Due to ascertainment bias, the proportion of cases of premature ovarian failure that are caused by this mechanism is unclear. Evidence is accumulating to suggest that autoimmune oophoritis and adrenal autoimmunity may represent a continuum of one pathophysiologic process. To determine the true prevalence of steroid cell autoimmunity as a mechanism of premature ovarian failure, it is necessary to evaluate prospectively a group of young women who are representative of the population at large. To this end, we have been recruiting young women with 46,XX spontaneous premature ovarian failure who meet two primary criteria: they are primarily concerned with infertility and amenorrhea, and they generally consider themselves to be in good health otherwise. In a prospective series, we found evidence for steroid cell autoimmunity in six of 123 women. In ongoing work, we are investigating the accuracy of steroid cell antibodies in predicting the presence of autoimmune oophoritis. We are collating the results of the antibody test with a gold standard: autoimmune oophoritis confirmed by ovarian biopsy.

We previously demonstrated that a low number of follicles is associated with follicle dysfunction in young women with 46,XX spontaneous premature ovarian failure and that the follicle dysfunction is mediated by inappropriate luteinization; the mechanism is related to inadequate negative feedback on gonadotropins due to a low follicle cohort size. For this reason, we are looking for gene mutations that might cause germ cell deficiency and low cohort size as a mechanism of premature ovarian failure. During the past year, we tested the hypothesis that mutations in the c-kit receptor may be the cause of the reduction in germ cell number in these women. Mice with such mutations have a reduced number of germ cells. The c-kit system, including c-kit receptor and its ligand, is known to play important regulatory roles in ovarian folliculogenesis and oogenesis. We found that mutations in the coding region of the kit receptor gene are uncommon in women with premature ovarian failure. In ongoing work, we are evaluating the kit ligand gene.

### Antigenic Role of MATER in Autoimmune Premature Ovarian Failure

*Vanevski, Tong, Nelson*

Although autoantibodies develop in most autoimmune diseases, specific ovarian antigens involved in human autoimmune premature ovarian failure have yet to be confirmed. Autoimmune premature ovarian failure causes young women to develop amenorrhea, menopausal symptoms, and infertility. A similar syndrome appears in mice with post-thymectomy autoimmune oophoritis. We have been developing both a serum marker that will identify women with premature ovarian failure attributable to autoimmune oophoritis and a treatment that will restore fertility. We have demonstrated that mice with autoimmune oophoritis develop antibodies against MATER. Experiments are under way to test the antigenic role of MATER in the development of autoimmune premature ovarian failure in mice.

## SELECTED PUBLICATIONS

1. Alzubaidi NH, Chapin HL, Vanderhoof VH, Calis KA, Nelson LM. Meeting the needs of young women with secondary amenorrhea and spontaneous premature ovarian failure. Obstet Gynecol. 2002;99:720-725.

2. Bakalov VK, Vanderhoof VH, Bondy CA, Nelson LM. Adrenal antibodies detect asymptomatic autoimmune adrenal insufficiency in young women with spontaneous premature ovarian failure. Hum Reprod. 2002;17:2096-2100.
3. Nelson LM. Autoimmune ovarian failure: comparing the mouse model and the human disease. J Soc Gynecol Investig. 2001;8(1 Suppl Proceedings):S55-77.
4. Shibanuma K, Tong ZB, Vanderhoof VH, Vanevski K, Nelson LM. Investigation of KIT gene mutations in women with 46,XX spontaneous premature ovarian failure. BMC Womens Health. 2002;2:8.
5. Tong ZB, Bondy CA, Zhou J, Nelson LM. A human homologue of mouse *Mater*, a maternal effect gene essential for early embryonic development. Hum Reprod. 2002;17:903-911.

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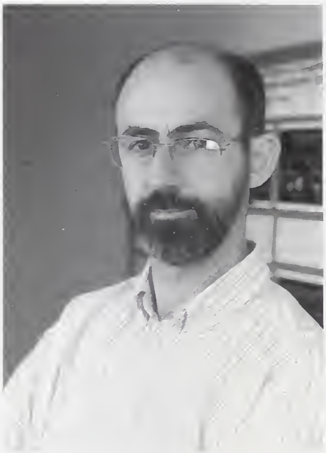
# PHOSPHOINOSITIDE-CALCIUM SIGNALING IN CELL REGULATION



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We investigate signal transduction pathways that mediate the actions of hormones and growth factors in mammalian cells, with special emphasis on the role of phosphoinositide-derived messengers. Current studies are aimed at (1) understanding the function and regulation of several phosphatidylinositol (PI) 4-kinases in the control of the synthesis of hormone-sensitive phosphoinositide pools; (2) characterizing the structural features that determine the catalytic specificity and inhibitor sensitivity of PI 3- and PI 4-kinases; (3) defining the molecular basis of protein-phosphoinositide interactions via the pleckstrin homology domain and other domains of selected regulatory proteins; (4) developing tools to analyze inositol lipid dynamics in living cells; and (5) determining the importance of lipid-protein interactions in the activation of cellular responses by G protein–coupled receptors and receptor tyrosine kinases.

## Characterization of a New Member of the Type-II Phosphatidylinositol 4-Kinase Family

*Balla A, Tuymetova, Barshishat, Balla T*

Phosphorylation of phosphatidylinositol (PI) to PI 4-phosphate is one of the key reactions in the production of phosphoinositides, which are lipid regulators of several cellular functions. The reaction is catalyzed by multiple enzymes that belong to either the type-II or type-III family of PI 4-kinases depending on the distinct catalytic properties of the two groups of enzymes. Type-III enzymes are structurally similar to PI 3-kinases and are sensitive to PI 3-kinase inhibitors, and two such enzymes have been cloned and characterized. Relatively little is known about the functions of the type-II PI4Ks in spite of extensive biochemical studies demonstrating their presence in a number of membrane compartments and organelles. The molecular identity of the type-II enzyme was recently revealed when the enzyme was cloned, based on purification of the protein from secretory granules by Barylko *et al.* at the University of Texas Southwestern Medical Center. Because of its dissimilarity to any known lipid or protein kinases, the type-II PI 4-kinase enzyme defines a novel enzyme family.

By searching the database for homologs of the recently published type-II PI4K, we identified a protein sequence with significant homology to the type-II PI4K enzyme. We termed this sequence PI 4-kinase type-IIβ in order to distinguish it from the previously cloned type-IIα enzyme. Based on Northern analysis, transcripts for both enzymes showed a relatively uniform tissue distribution with only a few notable differences, such as the prominent abundance of type-IIβ, but not type-IIα, mRNA in liver, and the relatively low level of type-IIβ mRNA in the brain and peripheral leukocytes. Characterization of the catalytic activities of the two enzymes showed that both enzymes are bona fide type-II PI 4-kinases, but type-IIβ was less active than type-IIα. Analysis of the intracellular distribution of the two enzymes in COS-7 cells revealed prominent association of both enzymes with the endosomal compartments. In addition, a significant amount of type-IIα, but only a small fraction of type-IIβ, was associated with the plasma membrane (Fig. 6). Catalytically inactive mutant forms of the enzymes (kinase-inactive proteins) showed more prominent plasma membrane localization and the accumulation of numerous vesicles in the juxtanuclear region of the cell, especially in the case of the type-IIα form. In addition, small tubular structures were observed in some of the cells expressing a high level of the kinase-inactive proteins, which were much more pronounced in the case of the inactive type-IIβ enzyme. Co-localization experiments have shown that during their trafficking both the nutrient transferrin receptor and the G protein–coupled $AT_1$ angiotensin receptor pass through endosomes positive for type-II PI 4-kinase isoforms. Expression of the kinase-inactive forms of the proteins was found to interfere with the rate of transferrin receptor endocytosis, indicating a functional role of the enzymes in the endocytic process.

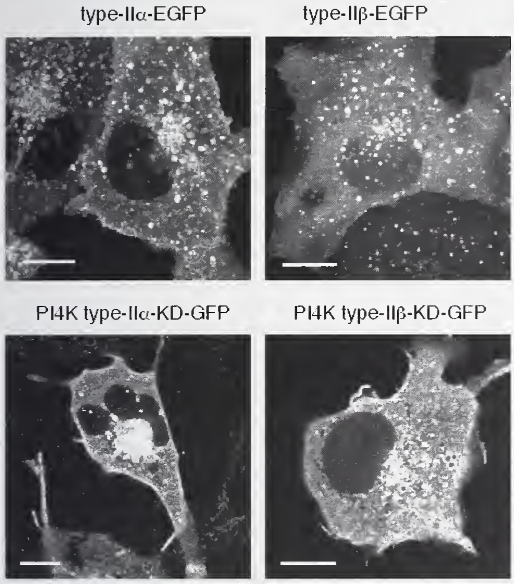

FIGURE 6

Cellular distribution of PI 4-kinase type-IIα and type-IIβ (upper panels) or their kinase-inactive mutant forms D308A and D304A of type-IIα and –IIβ (lower panels), respectively, expressed in COS-7 cells as GFP fusion proteins. Confocal images; bar represents 10 µm.

### Trafficking Pattern of Internalized $AT_1$ Angiotensin Receptors: Identification of PI 3-Kinase–Dependent and –Independent Pathways during $AT_1$ Receptor Recycling

*Balla T; in collaboration with Hunyady, Baukal, Gáborik, Lodge, Catt*

Activation of G protein–coupled receptors (GPCRs) results in the sequestration of the receptors in clathrin-coated pits in the plasma membrane followed by endocytosis of the ligand-receptor complex. After endocytosis of most GPCRs, most of the receptor is recycled back to the cell surface, although some of the receptor and most of the ligand are degraded via lysosomal or proteosomal degradative pathways. Little is known about the pathways involved in GPCR trafficking and even less about the molecular mechanisms directing the receptors toward the various vesicular trafficking routes. The involvement of phosphoinositides at numerous steps in vesicular trafficking is well documented in all species from yeast to mammals.

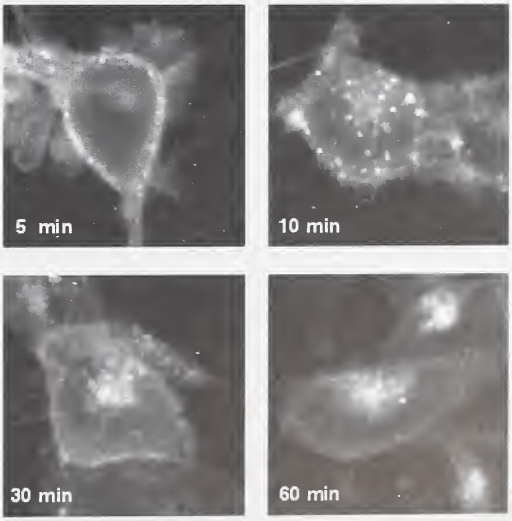

FIGURE 7

Internalization of $AT_1$ angiotensin receptors in HEK 293 cells stably expressing an $AT_1$-R-GFP chimeric construct. Confocal images of cells stimulated by angiotensin II for the indicated times.

To study $AT_1$-R trafficking and the role of phosphoinositides, we created stable HEK 293 cell lines expressing either a HA-tagged $AT_1$-R receptor or an $AT_1$-R with a GFP molecule fused to its C-terminus. To follow the ligand, we employed rhodamine-labeled angiotensin II (Rhod-Ang II) and identified the various endocytic compartments by using GFP-fused Rab proteins or GFP-fused protein domains that recognize PI(3)P. After stimulation with Ang II, the receptor and its ligand co-localized with rab5 and rab4 in early endosomes and subsequently with rab11 in pericentriolar recycling endosomes. Inhibition of PI 3-kinase by wortmannin (WT) or LY294002 caused the formation of large endosomal vesicles of heterogeneous Rab composition, which contained the ligand-receptor complex in their limiting membranes and in small vesicular structures associated with the large vesicles. In WT-treated cells, Alexa-transferrin (transferrin labeled with a different fluorophore) was found in small vesicles associated exclusively with the outside of large vesicles, while Rhod-Ang II also segregated into small vesicles in the lumen of the larger endosomes. WT treatment did not affect the late appearance of either Alexa-transferrin or Rhod-Ang II in pericentriolar recycling endosomes. In cells labeled with $^{125}$I-Ang II, WT treatment did not impair the rate of receptor endocytosis, but it significantly reduced the initial phase of receptor recycling without affecting its slow component. Similarly, WT inhibited the early but not the slow component of the recovery of $AT_1R$ at the cell surface after termination of Ang II stimulation. Our data indicate that internalized $AT_1$ receptors are processed via vesicles that resemble multivesicular bodies and recycle to the cell surface by a rapid PI 3-kinase–dependent recycling route as well as by a slower pathway that is less sensitive to PI 3-kinase inhibitors and that proceeds via the pericentriolar recycling endosomal compartment.

## SELECTED PUBLICATIONS

1. Balla A, Tuymetova G, Barshishat M, Geiszt M, Balla T. Characterization of type-II phosphatidylinositol 4-kinase isoforms reveals association of the enzymes with endosomal vesicular compartments. J Biol Chem. 2002;277:20041-20050.
2. Balla T, Varnai P. Visualizing cellular phosphoinositide pools with GFP-fused protein-modules. Science STKE. 2002;125:PL3.
3. Bondeva T, Balla A, Várnai P, Balla T. Structural determinants of Ras-Raf interaction analyzed in live cells. Mol Biol Cell. 2002;13:2323-2333.
4. Halet G, Tunwell R, Balla T, Swann K, Carroll J. The dynamics of plasma membrane PtdIns(4,5)$P_2$ at fertilization of mouse eggs. J Cell Sci. 2002;115:2139-2149.
5. Hunyady L, Baukal AJ, Gáborik Z, Olivares-Reyes AJ, Bor M, Szaszák M, Lodge R, Catt KJ, Balla T. Differential PI 3-kinase dependence of the early and late recycling of the G protein-coupled $AT_1$ angiotensin receptor. J Cell Biol. 2002;157;1211-1222.
6. Varnai P, Lin X, Lee SB, Tuymetova G, Bondeva T, Spat A, Rhee SG, Hajnoczky G, Balla T. Inositol lipid binding and membrane localization of isolated pleckstrin homology domains. Studies on the PH-domains of PLCdelta 1 and p130. J Biol Chem. 2002;277:27412-27422.

## COLLABORATORS

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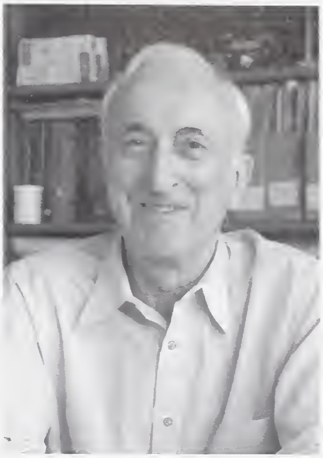
# PEPTIDE HORMONE RECEPTORS AND SIGNAL TRANSDUCTION



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We investigate the mechanisms by which peptide hormones control the activities of endocrine and other target cells. Major areas of interest include the characterization of receptors, signal transduction pathways, and other cellular processes involved in the neural control of gonadotropin-releasing hormone (GnRH) biosynthesis and secretion, the regulation and structure-function properties of receptors for GnRH and angiotensin II (Ang II), and the actions of GnRH and Ang II on target cells in the hypothalamus, pituitary, adrenal gland, and liver. Current research concerns the structural features and signal transduction pathways of the GnRH and Ang II receptors, and the manner in which these mechanisms regulate the secretory, metabolic, and growth responses of hypothalamic, pituitary, adrenal, and hepatic cells.

### The GnRH Pulse Generator: Role of Agonist-Induced Switching of G Protein Signaling

*Krsmanovic, Martinez-Fuentes, Navarro, Mores, Catt*

The pulsatile secretory activity of the GnRH-producing neuronal network in the hypothalamus, and consequently of pituitary gonadotroph cells, is essential for the maintenance of normal patterns of gonadotropin secretion and reproductive function. Based on the capacity of cultured fetal hypothalamic cells and immortalized GnRH neurons (GT1-7 cells) for prolonged pulsatile release of GnRH, we have analyzed the cellular and biochemical mechanisms of episodic neurosecretion *in vitro*. Pulsatile GnRH secretion is highly calcium-dependent and is stimulated by increased cell excitability and cyclic AMP (cAMP). The GnRH secretory profile is also influenced by agonist activation of the endogenous GnRH receptor (GnRH-R), which couples to $G_{q/11}$, as indicated by reduction in membrane-bound $\alpha_{q/11}$ and increased inositol phosphate/$Ca^{2+}$ signaling. In contrast, GnRH antagonists abolish pulsatile GnRH secretion and increase membrane-associated $\alpha_{q/11}$. GnRH stimulates cAMP production at low nanomolar concentrations but, at high concentrations, has an inhibitory effect that is abolished by pertussis toxin (PTX). Coupling of the GnRH-R to both $G_s$ and $G_i$ proteins was also indicated by the ability of nanomolar GnRH concentrations to reduce membrane-associated $\alpha_s$ and $\alpha_{i3}$ levels and of high concentrations to diminish $\alpha_{i3}$ levels. Conversely, $\alpha_{i3}$ was increased during GnRH antagonist and PTX treatment, with concomitant loss of pulsatile GnRH secretion. In cholera toxin (CTX)-treated GnRH neurons, decreases in $\alpha_s$ immunoreactivity and increases in cAMP production paralleled the responses to nanomolar GnRH concentrations. It is noteworthy that treatment with CTX and 8-bromo-cAMP amplifies episodic GnRH pulses but does not affect their frequency. These findings indicate that an agonist concentration–dependent switch in coupling of the GnRH-R between specific G proteins regulates $G_{q/11}$-$InsP_3$/$Ca^{2+}$ signaling as well as $G_s$-cAMP–stimulatory and $G_i$-cAMP–inhibitory responses. In a proposed model, this mechanism serves as a timer to regulate the frequency of $Ca^{2+}$- and cAMP-dependent episodes of GnRH release (Fig. 8).

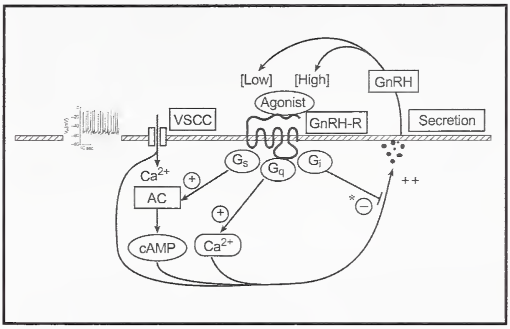

**FIGURE 8**

A proposed mechanism of pulsatile GnRH secretion. GnRH neuronal firing promotes calcium influx, activation of adenylyl cyclase and cAMP production, and calcium signaling, which stimulate GnRH secretion. At high local GnRH concentrations, an autocrine switch from Gs to Gi interrupts the rise in GnRH release. This is followed by a fall to baseline and subsequent reactivation of neurosecretion by the resurgent calcium/cAMP signaling pathways. AC, adenylyl cyclase.

### Regulatory Actions of Estrogen Receptors in GnRH Neurons

*Navarro, Krsmanovic, Murdock, Catt*

We have observed that hypothalamic GnRH neurons and their immortalized counterparts (GT1-7 cells) express not only nuclear but also cell membrane receptors for estrogen receptors (ERa and ERb). Both cell types exhibit positive immunostaining for plasma-membrane ERs as well as estradiol-induced changes in adenylyl cyclase activity. In GT1-7 cells, physiological (picomolar) estradiol concentrations cause a dose-dependent inhibition of cAMP production that is abolished by the ER antagonist ICI 182,780. Estradiol-induced inhibition of adenylyl cyclase in cells and membranes is also prevented by treatment with PTX, consistent with coupling of the membrane-bound estradiol receptors to an inhibitory G protein. In perifused GT1-7 cells and hypothalamic neurons, treatment with ovulatory-phase estradiol levels prolongs the GnRH interpeak interval, shortens peak duration, and increases peak amplitude. These findings have demonstrated that the membrane-associated ER expressed in GnRH neurons exhibits high-affinity interactions with adenylyl cyclase inhibitory G proteins by means of a rapid non-genomic mechanism, and modulates intracellular cAMP signaling and neuropeptide secretion. Recent studies have indicated that the negative regulatory action of estradiol on cAMP production is associated with a direct interaction between ERa and the $G_i$ a-subunit, as demonstrated by immunoprecipitation with a specific anti-ERa antibody. The agonist sensitivity of this interaction is commensurate with the low estradiol concentrations at which $G_i$-mediated inhibition of cAMP production is observed, suggesting that this process represents a physiological, negative feedback action of estrogen on the GnRH neuron.

### Angiotensin Receptor Structure, Activation, and Phosphorylation

*Olivares-Reyes, Shah, Catt; in collaboration with Hunyady, Balla*

The $AT_1$ angiotensin II (Ang II) receptor ($AT_1R$) is a typical G protein-coupled receptor (GPCR) and mediates the known physiological actions of the pressor octapeptide hormone, angiotensin II. Many such actions are mediated by coupling of the $AT_1R$ to $G_{q/11}$ proteins and by activation of phosphoinositide-calcium signaling and phosphorylation cascades that regulate cell growth, differentiation, and function. Several of the latter actions of Ang II are mediated by transactivation of receptor tyrosine kinases, in particular the EGF receptor (EGF-R), followed by activation of ras-dependent stimulation of MAP kinases. In the C9 hepatic cell line, the large share of Ang II–induced ERK phosphorylation depends on transactivation of the EGF-R, as described below. It has been proposed that the ability of certain GPCRs to activate MAP kinase depends on their agonist-induced phosphorylation, recruitment of arrestins, and clathrin-mediated endocytosis. However, studies on normal and mutant $AT_1$ receptors expressed in C9 and COS cells revealed that Ang II-induced ERK1/2 activation is independent of both $AT_1$-R and EGF-R endocytosis and is mediated by transactivation of the EGF-R. These and other studies have suggested that the dependence of MAP kinase activation on receptor endocytosis is confined to a minority of the GPCR family.

During agonist-induced endocytosis of the $AT_1$-R and many other GPCRs, invagination of clathrin-coated pits and vesicle formation depends on the recruitment of the 100 kDa GTPase, dynamin, and several other accessory proteins. An analysis of the roles of the functional domains of dynamin in endocytosis of the $AT_1$-R revealed that, similar to the recruitment of dynamin-1 during recycling of synaptic vesicles, interaction of the proline-rich domain of dynamin-2 with SH3 domains of amphiphysins and endophilins is essential for agonist-induced internalization of the $AT_1$ receptor. This mechanism could be of general importance in the dynamin-dependent endocytosis of other GPCRs in non-neural tissues. Related studies on the endocytosis and processing of the $AT_1$-R that used a GFP-tagged receptor expressed in HEK cells identified two phases of the internalization and recycling of the receptor to the cell membrane. The internalized $AT_1$ receptors are processed via vesicles that resemble multivesicular bodies and return to the cell surface by a rapid, PI 3 kinase–dependent recycling pathway as well as by a slower pathway that is less sensitive to inhibition of PI 3-kinase.

## Mechanisms of GPCR-Mediated Activation of MAP Kinase

*Shah, Farshori, Catt*

The ability of several agonist-activated GPCRs to stimulate MAP kinase activity and growth responses is mediated by a variety of intracellular pathways, including transactivation of growth factor receptors and their downstream signaling cascades to the nucleus. Our recent studies on agonist activation of endogenous $AT_1$ receptors expressed in hepatic C9 cells revealed that angiotensin-stimulated phosphoinositide hydrolysis, activation of PKCδ, and phosphorylation of the proline-rich tyrosine kinase Pyk-2 were associated with phosphorylation and activation of ERK1/2. These and related findings have demonstrated that Ang II increases the association of Pyk2 with Src and with the EGF receptor (EGF-R) and that the majority of Ang II-induced ERK phosphorylation depends on transactivation of the EGF-R. In these hepatic cells, Ang II–induced ERK activation is initiated by a PKCδ-dependent but $Ca^{2+}$-independent mechanism and is predominantly mediated by the Src/Pyk2 complex through transactivation of the EGF-R. Further investigations are addressing the nature of the interactions between GPCRs and receptor tyrosine kinases and the extent to which caveolae and other cell membrane structures are involved in the signaling cross-talk between different types of receptors expressed at the cell surface. Such studies are also in progress to evaluate the mechanisms and pathways that mediate the stimulation of MAP kinase responses in hypothalamic neuronal cells during activation of endogenous receptors for neurotransmitters, peptide hormones, and growth factors.

## SELECTED PUBLICATIONS

1. Gáborik Z, Szaszák M, Szidonya L, Balla B, Paku S, Catt KJ, Clark AJL, Hunyady L. b-arrestin- and dynamin-dependent endocytosis of the $AT_1$ angiotensin receptor. Mol Pharmacol. 2001;59:239-247.
2. García-Caballero A, Olivares-Reyes JA, Catt KJ, García-Saínz JA. Angiotensin $AT_1$ receptor phosphorylation and densitization in a hepatic cell line. Roles of protein kinase C and phosphoinositide 3-kinase. Mol Pharmacol. 2001;59:576-585.
3. Hunyady L, Baukal AJ, Gaborik Z, Olivares-Reyes JA, Bor M, Szaszak M, Lodge R, Catt KJ, Balla T. Differential PI 3-kinase dependence of early and late phases of recycling of the internalized $AT_1$ angiotensin receptor. J Cell Biol. 2002;157:1211-1222.
4. Krsmanovic LZ, Mores N, Navarro CE, Tomic M, Catt KJ. Regulation of $Ca^{2+}$-sensitive adenylyl cyclase in gonadotropin-releasing hormone neurons. Mol Endocrinol. 2001;15:429-440.

5. Olivares-Reyes JA, Smith RD, Hunyady L, Shah BH, Catt KJ. Agonist-induced signaling, desensitization, and internalization of a phosphorylation-deficient $AT_2$ angiotensin receptor. J Biol Chem. 2001;276:37761-37768.
6. Shah BH, Catt KJ. Calcium-independent activation of ERK1/2 MAP kinases by angiotensin II in hepatic C9 cells: roles of PKCd, Src/Pyk2, and EGF receptor transactivation. Mol Pharmacol. 2002;61:343-351.
7. Shah BH, Olivares-Reyes AJ, Yesilkaya A, Catt KJ. Independence of angiotensin II-induced MAP kinase activation from $AT_1$-receptor internalization in clone 9 hepatocytes. Mol Endocrinol. 2002;16:610-620.
8. Szaszak M, Gaborik Z, Turu G, McPherson PS, Clark AJ, Catt KJ, Hunyady L. Role of the proline-rich domain of dynamin-2 and its interactions with Src homology 3 domains during endocytosis of the $AT_1$ angiotensin receptor. J Biol Chem. 2002;277:21650-21656.

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# GONADAL RECEPTORS AND MECHANISM OF ACTION OF PEPTIDE HORMONES IN STEROIDOGENIC CELLS

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We investigate the molecular basis of peptide hormone control of gonadal function, with particular emphasis on the structure and regulation of luteinizing hormone and prolactin receptor genes, and the regulatory mechanism(s) involved in the hormonal control of steroidogenesis. Our studies focus on the regulation of the promoter domain of the LH receptor gene and its control by both nuclear orphan receptors (histone acetylation, deactylation, and methylation) and second messengers as well as on the complex genomic structure of the prolactin receptors (PRLR) and the multiple promoter control of their transcription. We are particularly interested in the function of two novel short forms of alternative spliced transcripts of the prolactin receptors and their relevance to physiological regulation and breast cancer. We also investigate hormone-regulated membrane coupling and intracellular events involved in the modulation of steroid biosynthesis in the testis and ovary as well as novel gonadotropin-regulated genes of relevance to testicular and ovarian function and other reproductive processes. Two such genes are an RNA helicase and a Long Chain Acyl CoA synthetase that are developmentally regulated and present in the Leydig cell and specific tubule cells. These genes are of interest for their potential relevance to steroidogenesis and stage-specific translation in spermatogenesis.

## Transcriptional Regulation of the LH Receptor

*Zhang, Dufau*

The luteinizing hormone receptor (LHR), a G protein–coupled receptor, is essential for reproductive function and is predominantly located in the plasma membrane of gonadal cells. It mediates gonadotropin signalling and triggers intracellular responses that participate in gonadal maturation and function as well as in the regulation of steroidogenesis and gametogenesis. The LHR gene is also expressed in several non-gonadal tissues, including the uterus and placenta, where its functions have not been determined. We previously demonstrated that the nuclear orphan receptors EAR2 and EAR3/COUP-TF1 (inhibitory) and TR4 (stimulatory) regulate the Sp1-/Sp3-driven TATA-less 180 bp promoter of the human LH receptor (hLRH). The orphan receptors bind competitively and with high affinity to an imperfect direct-repeat motif containing an estrogen response element half-site and a second degenerate half-site (DR) located within the promoter 5' of two functional Sp1/Sp3 sites. The orphan receptors exhibit differential binding to the rat and human LHR promoters. EAR2 and EAR3/COUP-TFI bind with two- to three-fold higher affinity to the human promoter than to the rat promoter as a consequence of the presence of a G 3' to the second DR site in the human sequence and, accordingly, exert weaker inhibition on rat promoter activity. In addition, TR4 is inactive in the rat promoter due to a single base pair mismatch in the second DR site that abolishes its binding. Changes in endogenous levels of EAR2 and EAR3 during gonadotropin stimulation of rat granulosa cells in culture correlate with derepression of promoter activity.

Modification of chromatin structure by histone deacetylases (HDACs) is an important mechanism in the modulation of eukaryotic gene transcription. Investigation of the regulation of the human LHR by histone deacetylases in human choriocarcinoma cells (JAR cells) showed that LHR transcription was markedly induced when histone deacetylase activity was inhibited by trichostatin A (TSA) and that histone deacetylation caused significant repression of the hLHR gene transcription in these cells. Acetylated histones H3 and H4 accumulated in TSA-treated cells and were shown to be predominantly associated with the LHR promoter (-181/+27 bp from ATG at +1) and only minimally with the adjacent 5' region (-489/-235 bp). In addition, TSA significantly enhanced the recruitment of RNA polymerase II to the promoter. This promoter-specific localization of histone acetylation could provide a more accessible environment for the recruitment of components(s) of the general transcriptional machinery and of RNA Pol II. We identified only one of the two Sp1 sites essential for basal promoter activity as critical for the TSA effect (Sp1-1 site, -79/-71 bp from ATG +1), but the binding of Sp1/Sp3 to the site remained unchanged in the absence or presence of TSA. The orphan receptor DR site did not participate in TSA induction. We used DNA precipitation assays (DAPA) to determine whether Sp1/Sp3 were candidates to target non–DNA binding proteins. Our findings demonstrated the formation of a multi-protein complex including DNA binding and non-binding protein(s). Using co-immunoprecipitation assays to determine the association order of the individual components, we demonstrated that the multi-protein complex was recruited to the LHR promoter via interaction with Sp1 and Sp3. HDAC1 and HDAC2 docked directly to Sp1-bound DNA and indirectly to Sp3-bound DNA through RbAp48 while mSin3A interacted with hDACs. Investigation of the mode of repression of hLHR gene transcription by the HDACs/mSin3A complex in co-transfection studies revealed that HDACs 1 and 2 strongly inhibited hLRH promoter activity induced by TSA. Transfection of mSin3A caused minor inhibition of hLHR promoter activity, presumably because of its cooperation with endogenous HDACs. HDAC1 and HDAC2 potently repressed hLHR gene transcription, and mSin3A potentiated the inhibition caused by HDAC1. These findings demonstrated that the HDAC-mSin3A complex has an important role in the regulation of hLHR gene transcription by interaction with Sp1/Sp3 and by region-specific changes in histone acetylation and Pol II recruitment within the hLHR promoter. We have identified the hLHR gene promoter as a target for regulatory repression by the HDACs-mSin3A complex during the control of hLHR gene transcription. Expression of the LHR gene is subject to tonic repression by deacetylation of its gene promoter. The regulated derepression of such inhibitory control of the LHR gene, through as yet unidentified signal inputs, may provide

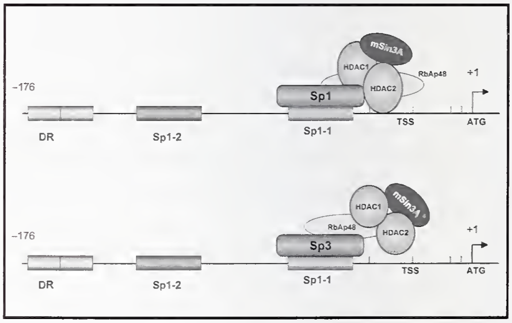

**FIGURE 9**

**Recruitment of HDACs-mSin3A Complex to the Human LHR Promoter**

Sp1-1 and Sp1-2 are functional sites for promoter activity. Both sites bind to Sp1 and Sp3 and contribute to basal promoter activity. The HDAC-mSin3A complex is recruited directly to Sp1 the site and indirectly through RbAp48 to Sp3 bound to this site. DR: imperfect estrogen receptor half-site direct repeat for binding of orphan receptors (OR) EAR2, EAR3/COUP-TF1 binding/function (inhibitory), and TR4 (stimulatory). TSS: transcriptional start sites (vertical lines).

functional regulation during the induction and cyclic variations in the differentiation, growth, and development of gonadal cells.

### Expression of a Novel Gonadotropin-Regulated Testicular RNA Helicase

*Sheng, Tsai-Morris, Dufau*

The gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) that we cloned from the rat Leydig cell, mouse, and human testis cDNA libraries is a novel member of the DEAD-box protein family of RNA helicases that possesses ATPase and RNA helicase activity; it is the first member found to be regulated by a hormone. The mRNA of the GRTH gene is transcriptionally up-regulated by gonadotropin (hCG) via cyclic AMP-induced androgen formation in testicular Leydig cells. Immunocytochemical analysis of GRTH protein levels revealed that GRTH protein is predominantly present in Leydig cells. It is also expressed to a lesser extent in germ cells of the adult rat; we identified the highest immunosignals in pachytene spermatocytes and round spermatids at stages VIII and IX. Western blot analysis displayed a 43 kDa GRTH protein band in Leydig cells, where its levels significantly increased at 24 hours and returned to control levels four days after *in vivo* treatment with hCG. The androgen receptor antagonist Flutamide markedly suppressed basal and hCG-induced expression of GRTH protein. We observed high expression of GRTH protein in pachytene spermatocytes while low levels were present in round spermatids. The compartmen-talized localization of GRTH in the tubules indicates that the protein may be involved in the meiotic process during spermatogenesis. Our studies have demonstrated marked up-regulation of GRTH protein expression by hCG via androgen formation in Leydig cells at agonist doses that cause down-regulation of LH receptors and steroidogenic enzymes. We have also shown that the sites of protein expression and pattern of regulation by hCG and androgen are highly correlated with the mRNA changes observed in our previous studies.

### Prolactin Receptors

*Meng, Leondires, Dong, Hu, Tsai-Morris, Dufau*

The polypeptide hormone prolactin has highly diversified biological actions in reproduction, lactation, maternal behavior, steroidogenesis, growth, metabolism, water-salt balance, and immune regulation. Its actions are mediated by a single-transmembrane domain receptor of the cytokine/growth hormone family. The receptor is widely distributed and exists in several forms, including long and intermediate forms, and two short forms with abbreviated cytoplasmic domains that inhibit the function of the long form. Prolactin receptors (PRLR) are expressed in normal and neoplastic human tissues and mediate prolactin-induced proliferative actions in human breast cancer cells that are additive to those of estrogen. Circulating prolactin of pituitary origin and that synthesized locally in the breast could contribute to tumor biogenesis. Adipose cells are a major source of estrogen in post-menopausal women and could exert paracrine control of prolactin and PRLR synthesis in the epithelial cells of the breast. Current evidence and the ability of prolactin to stimulate the growth of rodent mammary tumors indicate that prolactin could contribute to the development of such tumors and possibly to the development of human breast cancer.

To determine the control mechanism(s) underlying human PRLR gene expression, we defined the 5' exonic gene structure and initiated studies on promoter identification and regulation. The expression of the PRLR is controlled by a highly complex regulatory system at the transcriptional level. Of the three promoters used for transcription of non-coding exons-1 in the rat (PI, PII, and PIII) and the two in the mouse (PI and PIII), only PIII is shared with the human. Resolution of the complete genomic structure of the hPRLR gene revealed that transcription of the hPRLR is controlled by multiple promoters that use distinct mechanisms to regulate gene expression. The gene has five human-specific non-coding exons-1 ($hE1_{N1-5}$) as well as a generic exon-1 that is present in rodents and human-$hE1_3$. We identified all alternative first exons in a genomic fragment of a PAC clone that included the 5' UTR and the coding region of the hPRLR. Their order with their corresponding 5' flanking and promoter sequences (hPIII generic; $hP_{N1-5}$, human specific) was established in chromosome 5p14-13. We found that promoters used in the transcription of exon 1 generic species, $hE1_3$

(hPIII) and the human-specific $hE1_{N1}$ ($hPI_{N1}$), employ distinct mechanisms to control hPRLR transcription. In the human, Sp1/Sp3 are the major transcription factors that participate in hPIII promoter activation while C/EBPβ can minimally support activity when Sp1 is mutated. The $hP_{N1}$ promoter uses a distinct mechanism for controlling hPRLR transcription. Two putative regulatory complexes that specifically bind to Ets and NR elements may contribute to transcriptional activity; the transfactors involved remain to be determined. Our studies have excluded transfactors that are known members of the PEA3 or Fli1 /ERG or Ets subfamily for interaction with the ETS element. Furthermore, the complex formed at the NR half-site is unlikely to include any of the known monomeric orphan receptors; it does not contain the required 5' extended core motif required for high-affinity interaction with the individual monomer.

Estradiol ($E_2$) significantly increased the expression of both hPRLR mRNA transcripts $hE1_3$ and $hE1_{N1.}$ In all human tissues examined (ovary, testis, liver, T47D cells), the expression levels of these alternative first exons are markedly lower than that of the $hE1_3$. In both normal breast and cancer cells lines, hPIII appears to be the more commonly used promoter of hPRLR and is therefore the main focus of our ongoing investigation of the hormonal induction of hPRLR gene expression. In transfected T47D cells, $E_2$ activated the hPIII promoter in a dose-dependent manner, and such activation was sensitive to the specific estrogen receptor antagonist ICI 182,780. The lack of a formal ERE in these promoters suggests that the effect of estradiol is mediated through association of the activated ER with relevant DNA binding transfactor(s). These findings support the role of $E_2$ in the regulation of hPRLR expression in human breast cancer cell lines.

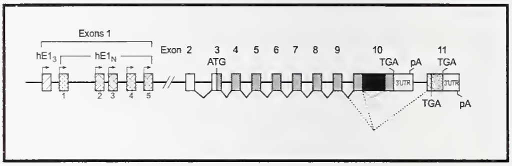

FIGURE 10

**Schematic Representation of the Prolactin Receptor Gene** The location of multiple first exons is indicated. All first non-coding exons are spliced onto the common non-coding exon 2 followed by the third exon in which translation starts at ATG. Exons 4 through 11 are coding exons. The long form of the receptor is encoded by exons 3 through 10. Exon 8 encodes the transmembrane domain and exon 10 most of the intracellular domain. The intermediate form is encoded by exons 3 through 10 and contains a partial deletion of exon 10 (black box). The two short forms (S1a and S1b) with truncated cytoplasmic domains are derived from alternative splicing of exons 10 and 11. S1a encodes 376 amino acids (aa) and contains partial exon 10 and a unique 39 aa C-terminal region derived from exon 11. S1b encodes a 288 aa protein that lacks exon 10 and contains three amino acids at the C-terminus derived from exon 11 (splice sites are indicated).

## SELECTED PUBLICATIONS

1. Dufau ML,Tsai-Morris CH, Tang PZ, Khanum A. Regulation of steroidogenic enzymes and a novel testicular RNA helicase. J Steroid Biochem Mol Biol. 2001;76:187-197.
2. Hu ZZ, Meng J, Dufau ML. Isolation and characterization of two novel forms of the human prolactin receptor generation by alternative splicing of a newly identified exon 11. J Biol Chem. 2001;276:41086-41093.
3. Hu ZZ, Zhuang L, Meng J, Tsai-Morris CH, Dufau ML. Complex 5' genomic structure of the human prolactin receptor: multiple alternative exons 1 and promoter utilization. Endocrinology. 2002;143:2139-2142.
4. Leondires MP, Hu ZZ, Dong J, Tsai-Morris CH, Dufau ML. Estradiol stimulates expression of two human prolactin receptor isoforms with alternative exons-1 in T47D breast cancer cells. J Steroid Biochem Mol Biol. 2002;82:263-268.
5. Zhang Y, Dufau ML. EAR2 and EAR3/COUP-TFI regulate transcription of the rat LH receptor. Mol Endocrinol. 2001;15:1891-1905.
6. Zhang Y, Dufau ML. EAR2 and EAR3/COUP-TFI regulate transcription of the rat luteinizing hormone receptor. Mol Endocrinol. 2001;15:1891-1905.
7. Zhang Y, Dufau ML Silencing of transcription of the human luteinizing hormone receptor gene by histone deacetylase-mSin3A complex. J Biol Chem. 2002;277:33431-33438.

# CALCIUM SIGNALLING AND CALCIUM-CONTROLLED CELLULAR FUNCTIONS

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We investigate the cellular signalling cascades in endocrine and neuroendocrine cells and the interactions between plasma membrane electrical events and receptor-controlled pathways. Our objective is to elucidate the mechanisms controlling calcium signalling and the role of calcium ions as messengers in control of signalling, secretion, and gene expression. Our approach has been to characterize calcium signalling from a biophysical, physiological, and pharmacological point of view. More recently, we have begun to use molecular biological approaches to identify pathways participating in the generation and control of calcium signals and to address how the calcium-signalling function in neuroendocrine cells is related to the structures of individual receptors and channels. Our current investigations focus on the calcium-signalling function of purinergic receptors, the mechanism of endothelin and thyrotropin-releasing hormone actions in pituitary lactotrophs, and the role of calcium as an extracellular and intracellular messenger.

### Calcium Signalling by Purinergic Receptors

*He, Tomic, Stojilkovic; in collaboration with Koshimizu*

Pituitary cells secrete ATP, which acts as an autocrine and/or paracrine extracellular messenger on two families of purinergic receptors termed P2YRs and P2XRs. P2YRs are G protein–coupled receptors, and P2XRs are cation-permeable channels that conduct calcium and facilitate voltage-sensitive calcium entry if expressed in excitable cells. Even though it is known that these channels vary with respect to their ligand preferences and channel kinetics during activation, desensitization, and recovery, the contributions of distinct receptor subdomains to the subtype-specific behavior have been incompletely characterized. We recently showed that the wild-type P2X2aR and its splice form lacking the intracellular Val$^{370}$-Gln$^{438}$ C-terminal sequence (P2X2bR) respond to ATP stimulation with comparable EC$_{50}$s and peak current/calcium responses but desensitize in a receptor-specific manner: P2X2aR desensitizes slowly and P2X2bR desensitizes rapidly.

We studied the effects of different agonists and of substituting the ectodomain on the pattern of calcium signalling by P2X2aR and P2X2bR. Both receptors showed similar EC$_{50}$s (estimated from the peak current/calcium response) and IC$_{50}$s (estimated from the rate of current/calcium signal desensitization) for agonists in the order 2-MeS-ATP $\leq$ ATP $\leq$ ATP-$\gamma$-S < BzATP << $\alpha\beta$-methylene ATP; the IC$_{50}$s for agonists shifted to the right compared with their EC$_{50}$s. Furthermore, the ATP-induced receptor-subtype–specific pattern of desensitization was mimicked by high- but not low-efficacy agonists.

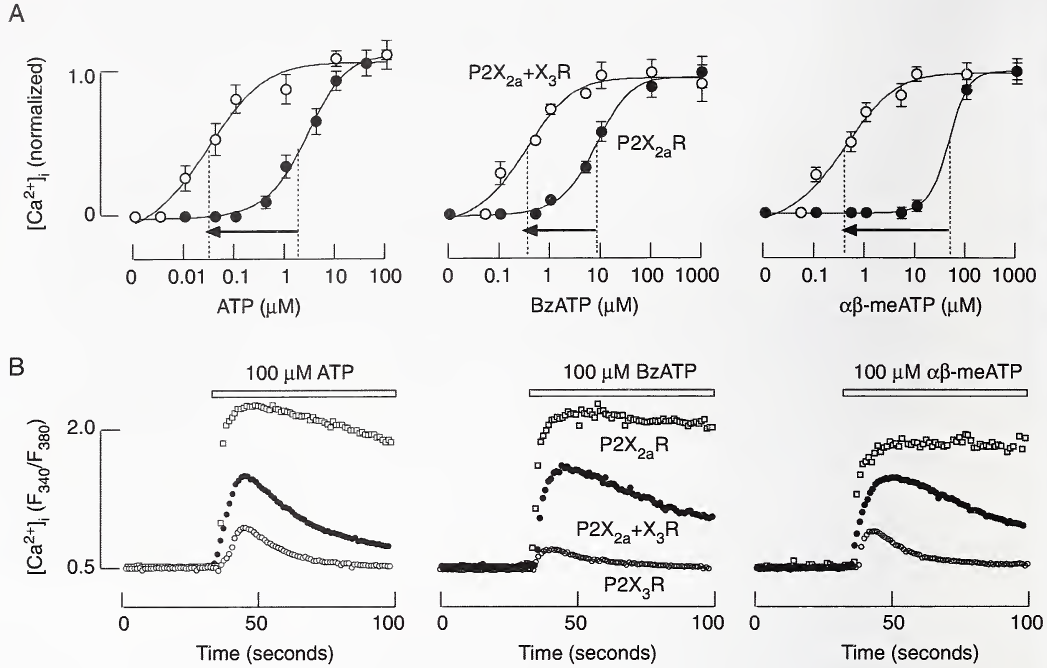

**FIGURE 11**

Influence of the substitution of ectodomain at P2X2Rs on agonistic potency of ATP (left panels), BzATP (central panels), and αβ-methylene ATP (right panels). A, Change in the EC$_{50}$s for agonists in cells expressing P2X$_{2a}$+X$_3$Rs. B, Comparison of the peak amplitude of [Ca$^{2+}$]$_i$ signals in P2X2a+X3R-expressing cells. P2X2b+X3Rs showed comparable leftward shifts in EC$_{50}$s for three agonists and a decrease in peak amplitude of calcium responses.

These results suggested a ligand-specific desensitization pattern, which we sought to confirm by generating chimeric P2X2aR and P2X2bR containing the Val$^{60}$-Phe$^{301}$ ectodomain sequence of P2X3R and the Val$^{61}$-Phe$^{313}$ ectodomain sequence of P2X7R instead of the native Ile$^{66}$-Tyr$^{310}$ sequence. The homomeric receptors bearing the extracellular domain of the P2X3 subunit in the P2X2a-based backbone mimicked two intrinsic functions of P2X3R: sensitivity to αβ-methylene ATP and ecto-ATPase–dependent recovery from endogenous desensitization; the two functions were localized to the N- and C-terminal halves of the P2X$_3$R extracellular loop, respectively. Furthermore, the mutated P2X2a+X3R and P2X2b+X3R exhibited comparable EC$_{50}$s for ATP, BzATP, and αβ-methylene ATP in the submicromolar concentration range and desensitized in a receptor-specific and ligand-nonspecific manner. On the other hand, the chimeric P2X2+X7R exhibited decreased sensitivity for ATP and desensitized in a receptor-nonspecific manner. The results suggest that the efficacy of agonists for the ligand-binding domain of P2X2Rs reflects the strength of desensitization controlled by their C-terminal structures.

We have also analyzed the agonist selectivity and desensitization rates of P2XRs in heteromeric configuration. First, we demonstrated the physical and functional heteromerization of native and mutant P2X2a and P2X2b subunits. In heteromeric receptors, the ectodomain of P2X3 was a structural determinant for ligand selectivity and recovery from desensitization, and the C-terminus of P2X2R was an important factor for the desensitization rate. Furthermore, [γ-$^{32}$P]8-azido ATP, a photoreactive agonist, was effectively cross-linked to the P2X3 subunit in homomeric receptors but not in heteromeric P2X2+P2X3Rs. The results indicate that heteromeric receptors formed by distinct P2XR subunits develop new functions resulting from integrative effects of the participating extracellular and C-terminal subdomains.

### Receptor-Controlled Action Potential Secretion Coupling

*Tomic, He, Van Goor,*[a] *Stojilkovic*

Two $Ca^{2+}$-mobilizing receptors expressed in lactotrophs, endothelin-A (ET-A) and thyrotropin-releasing hormone (TRH), induce both rapid calcium release from intracellular stores and prolactin secretion but differ in their actions during sustained stimulation. TRH facilitates and ET-1 inhibits voltage-gated calcium influx and prolactin secretion. ET-1-induced the depolarization of cells, and the enhancement of calcium influx upon calcium mobilization was established in both quiescent and firing lactotrophs treated overnight with pertussis toxin. Activation of adenylyl cyclase by forskolin and the addition of cell permeable 8Br-cAMP did not affect the ET-1–induced sustained inhibition of voltage-gated calcium influx, suggesting that the cAMP-protein kinase A signalling pathway does not mediate the inhibitory action of ET-1 on calcium influx.

Consistent with the role of pertussis toxin–sensitive potassium channels in ET-1–induced hyperpolarization of controls but not pertussis toxin-treated cells, ET-1 decreased the cell input resistance and activated a cesium-sensitive potassium current. The addition of cesium also mimicked the action of pertussis toxin on the pattern of sustained calcium signalling, but only in about half of ET-1–stimulated cells, and did not change ET-1–induced inhibition of prolactin secretion. We observed similar effects of pertussis toxin and cesium in immortalized GH3 cells transiently expressing ET-A receptors. Moreover, the following blockers did not affect the agonist-specific patterns of calcium signalling and prolactin secretion: apamin and paxilline, specific blockers of calcium-activated SK- and BK-type potassium channels, respectively; E-4031, a blocker of the *ether a-go-go* potassium channel; and linopirdine, a blocker of the M-type potassium channel. The results suggest that ET-1 inhibits voltage-gated calcium influx through activation of cesium-sensitive channels, presumably the Gi/o-controlled inward rectifier potassium channels, and that the agonist also inhibits prolactin release, but downstream of calcium influx. Further studies are required to identify the mechanism of sustained TRH-induced facilitation of voltage-gated calcium influx and prolactin secretion.

### Extra- and Intracellular Messenger Functions of Calcium in Pituitary Cells

*Andric, Kostic, Tomic, Zivadinovic,*[b] *Stojilkovic*

It is well established that G protein–coupled receptors stimulate nitric oxide–sensitive soluble guanylyl cyclase by increasing intracellular calcium and activating calcium-dependent nitric oxide synthases. In pituitary cells, receptors that stimulated adenylyl cyclase, growth hormone–releasing hormone, corticotropin-releasing factor, and thyrotropin-releasing hormone also stimulated calcium signalling and increased cGMP levels, whereas receptors that inhibited adenylyl cyclase, endothelin-A and dopamine-2, also inhibited spontaneous calcium transients and decreased cGMP levels. However, abolition of calcium signalling did not block receptor-controlled up- and down-regulation of cyclic nucleotide accumulation, suggesting that cAMP production affects cGMP accumulation. We observed agonist-induced cGMP accumulation in cells incubated in the presence of various phosphodiesterase and soluble guanylyl cyclase inhibitors, confirming that Gs-coupled receptors stimulated *de novo* cGMP production. In addition, cholera toxin (an activator of Gs), forskolin (an activator of adenylyl cyclase), and 8-Br-cAMP (a permeable cAMP analog) mimicked the stimulatory action of Gs-coupled receptors on cGMP production. Basal, agonist-, cholera toxin–, and forskolin-stimulated cGMP production, but not cAMP production, was significantly reduced in cells treated with H89, a protein kinase A inhibitor. The results indicate that coupling of seven plasma membrane–domain receptors to an adenylyl cyclase signalling pathway provides an additional calcium-independent and cAMP-dependent mechanism for modulating soluble guanylyl cyclase activity in pituitary cells.

Calcium can serve not only as an intracellular messenger but also as an extracellular messenger controlling the gating properties of plasma membrane channels: it also acts as an agonist for G protein-coupled calcium-sensing receptors. We studied the potential extracellular messenger functions of this ion in anterior pituitary cells. Depletion and repletion of extracellular calcium concentration–induced transient elevations in the intracellular calcium concentration and elevations in extracellular calcium above physiological levels decreased intracellular calcium in somatotrophs and lactotrophs. but not in gonadotrophs. The amplitudes and duration of intracellular calcium signals depended on the extracellular calcium concentration and its rate of change. which resulted exclusively from modulation of spontaneous voltage-gated calcium influx. Changes in extracellular calcium concentration also affected growth hormone and prolactin secretion. The prolactin secretory profiles paralleled the intracellular calcium profiles in lactotrophs. whereas growth hormone secretion was also stimulated by extracellular calcium independently of the status of voltage-gated calcium influx. Extracellular calcium modulated growth hormone secretion in a dose-dependent manner. with $EC_{50}$s of 0.75 and 2.25 mM. and with minimum secretion at about 1.5 mM. In a parallel experiment. cAMP accumulation progressively increased with elevation in extracellular calcium. whereas inositol phosphate levels remained unaffected. The results indicate the cell type–specific role of extracellular calcium in the control of calcium signalling and secretion.

## SELECTED PUBLICATIONS

1. Andric SA. Kostic TS. Tomic M. Koshimizu T. Stojilkovic SS. Dependence of soluble gyanlylyl cyclase activity on calcium signaling in pituitary cells. J Biol Chem. 2001:276:844-849.
2. He M-L. Koshimizu T. Tomic T. Stojilkovic SS. Purinergic P2$X_2$ receptor desensitization depends on coupling between ectodomain and C-terminal domain. Mol Pharmacol. 2002:62:1187-1197.
3. Koshimizu T. Ueno S. Tanoue A. Yanagihara N. Stojilkovic SS. Tsujimoto G. Hetermultimerization modulates PX receptor functions through participating extracellular and C-terminal subdomains. J Biol Chem. 2002:277:46891-46899.
4. Kostic TS. Andric SA. Stojilkovic SS. Spontaneous and receptor-controlled soluble guanylyl cyclase activity in anterior pituitary cells. Mol Endocrinol. 2001:15:1010-1022.
5. Kostic TS. Tomic M. Andric SA. Stojilkovic SS. Calcium-independent and cAMP-dependent modulation of soluble guanylyl cyclase activity by G protein-coupled receptors in pituitary cells. J Biol Chem. 2002:277:16412-16418.
6. Tomic M. Van Goor F. He M-L. Zivadinovic D. Stojilkovic SS. $Ca^{2+}$-mobilizing endothelin-A receptors inhibit voltage-gated $Ca^{2+}$ influx through $G_{i/o}$ signaling pathway in pituitary lactotrophs. Mol Pharmacol. 2002:61:1329-1339.
7. Van Goor F. Li Y-X. SS Stojilkovic. Paradoxical role of large-conductance calcium-activated $K^+$ (BK) channels in controlling action potential-driven $Ca^{2+}$ entry in anterior pituitary cells. J Neurosci. 2001:15:5902-5915.
8. Van Goor F. Zivadinovic D. Martinez-Fuentes AJ. Stojilkovic SS. Dependence of pituitary hormone secretion on the pattern of spontaneous voltage-gated calcium influx: cell-type specific action potential secretion coupling. J Biol Chem. 2001:276:33840-33846.
9. Van Goor F. Zivadinovic D. Stojilkovic SS. Differential expression of ionic channels in rat anterior pituitary cells. Mol Endocrinol. 2001:15:1222-1236.
10. Zivadinovic D. Tomic M. Yuan D. Stojilkovic SS. Cell-type specific messenger functions of extracellular calcium in the anterior pituitary. Endocrinology. 2002:143:445-455.

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# STRUCTURE AND FUNCTION OF PEPTIDE AND PROTEIN HORMONES

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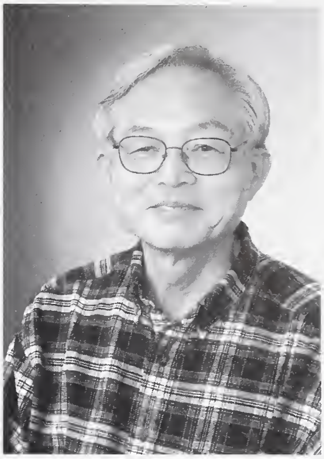

Our research program is concerned with the analysis, synthesis, expression, and structure-function relationships of peptide and protein hormones, with current emphasis on the structure and function of the angiotensin II (Ang II) and gonadotropin-releasing hormone (GnRH) receptors.

## Synthesis of Fluorescent Gonadotropin-Releasing Hormone Agonist

*Morell, Chen H-C*

Recently, we completed the milligram-scale synthesis and purification of a sulforhodamine 101 (Texas Red) conjugate of [D-Lys$^6$, des Gly$^{10}$amide]GnRHethylamide at ε-amino group of D-Lys$^6$. The reaction of [D-Lys$^6$, des-Gly$^{10}$amide]GnRHethylamide with N-succinimidyl ester of sulforhodamine 101 yielded five components as revealed by reverse-phase high-performance liquid chromatography in which the major component was characterized as the mono-sulforhodamine 101 acylated [D-Lys$^6$, des Gly$^{10}$amide]GnRHethylamide at ε-amino group of D-Lys$^6$. As revealed in confocal microscopy, this major conjugate is biologically active as a consequence of its ability to bind to the GnRH receptor and to internalize in the same manner as the GnRH agonist.

## Human Angiotensin II Type 1 Receptor Binding Proteins

*Chen H-D, Chen H-C*

In studies on the structure and function of the Ang II receptor, we demonstrated *in vivo* the association of hAT1 and the MHC class II–associated invariant chain Ii by yeast mating experiments and by co-localization at the plasma membrane of human embryonic kidney (HEK-293) cells on confocal microscopy. For co-localization studies, we transfected HEK cells with both AT1-green fluorescent protein and Ii-red fluorescent protein fusion proteins. In this cell line, we demonstrated inhibition of hAT1 receptor internalization in response to Ang II stimulation in the presence of Ii on the plasma membrane. The manner in which the Ii chain interferes with agonist-induced endocytosis of the AT1 receptor is under further investigation.

## Tandem Mass Spectrometric Sequencing of Phosphopeptides

*Li, Wang, Chen H-C*

We have also developed a chemical method that converts phospho-Ser and phospho-Thr to cysteic acid and β-methyl cysteic acid, respectively, for direct sequencing of the Ser and Thr phosphorylation sites by electron-spray ionization (ESI) tandem mass spectrometry. We modified and then, using an ESI-quadrupole ion trap mass spectrometer, sequenced five model phosphopeptides, including three synthetic P-Ser–, P-Thr–, or both P-Ser– and P-Thr–containing peptides; a protein kinase C–phosphorylated bovine myelin basic protein residues 4-14 peptide; and a phosphopeptide derived from β-casein trypsin digests. Following incubation of P-Ser or P-Thr containing peptides with $Na_2SO_3$/NaOH, amino acid analysis revealed that 90 percent P-Ser and 80 percent P-Thr were converted to cysteic acid and β-methyl cysteic acid, respectively. The conversion can be carried out at 1 μM concentration of the peptide. Both cysteic acid and β-methyl cysteic acid residues in the sequence were shown to be stable and easily identifiable in a series of y and b ions under general conditions for tandem mass spectrometric sequencing applicable to common peptides.

## PUBLICATION

Li W, Boykins RA, Backlund PS, Wang G, Chen H-C. Identification of phospho-serine and phospho-threonine as cysteic acid and beta-methylcysteic acid residues in peptides by tandem mass spectrometric sequencing. Anal Chem. 2002;74:5701-5710.

## COLLABORATORS

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# SIGNAL TRANSDUCTION IN SYNAPTIC TRANSMISSION

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We investigate the signal transduction mechanisms involved in the enhancement of synaptic transmission and plasticity. Studies of these neural processes are essential to understanding the complex problems of learning and memory. Our approach is to generate genetically modified mice by deletion or expression of a gene specifically expressed in the brain. We have generated a strain of mice devoid of a neural-specific protein, neurogranin (Ng). Ng is normally expressed at a high level in the neurons within the cerebral cortex, hippocampus, and amygdala; it has been implicated in the modulation of synaptic plasticity. The multiple effects of Ng could account for the positive impact of the protein on the behavior of mice.

## Roles of Neurogranin in Enhancing the Signalling for Hippocampus-Dependent Learning and Memory

*Huang F, Li, Wu, Huang K*

Ng binds to calmodulin (CaM) in an inverse $Ca^{2+}$-sensitive manner; that is, its binding affinity for CaM decreases with increasing $Ca^{2+}$ concentration. It is believed that, at basal levels of $Ca^{2+}$, all CaM is sequestered by Ng, whose cellular concentration is at least twice that of CaM. Upon synaptic stimulation, the influxed $Ca^{2+}$ displaces Ng from the Ng/CaM complex to form $Ca^{2+}$/CaM. The buffering of CaM by Ng serves as a mechanism to regulate neuronal free $Ca^{2+}$ and $Ca^{2+}$/CaM concentrations. Furthermore, Ng is readily phosphorylated by protein kinase C (PKC) and oxidized by nitric oxide (NO). Both the phosphorylated and oxidized Ng exhibit lower affinities for CaM than the unmodified protein; thus, the modifications of Ng extend the availability of CaM even after the intracellular level of $Ca^{2+}$ is reduced to basal levels. Synaptic responses triggering long-term potentiation (LTP) or long-term depression (LTD) depend on the amplitude of $Ca^{2+}$ influx and the sensitivity of the transduction machinery to amplify the signal. Based on our previous finding that the autophosphorylation of $Ca^{2+}$/CaM-dependent protein kinase II was enhanced by Ng, we speculate that other $Ca^{2+}$- and $Ca^{2+}$/CaM-regulated pathways are also upregulated by neurogranin.

Ng is highly concentrated in the cell bodies and dendrites of selective neurons within the cerebral cortex, hippocampus, and amygdala. To examine the role of Ng in neural function, we generated mutant mice devoid of Ng. The Ng knock-out (KO) mice did not exhibit any obvious developmental or neuroanatomical abnormalities but showed impairment of spatial learning and memory. These deficits in the KO mice were accompanied by a defective mechanism in the activation of $Ca^{2+}$/CaM-dependent protein kinase II, whose activation by autophosphoryla-tion has been linked to the postsynaptic mechanism for the induction of LTP and storage of long-term memory. The concentration of hippocampal Ng in adult wild-type mice is one of the highest of the known neuronal CaM-binding proteins. Induction of LTP caused a rapid phosphorylation of the protein in the hippocampal CA1 region. Testing with the Morris water maze showed significant relationships between the levels of hippocampal Ng among $Ng^{+/-}$ mice and their performances; however, such relationships were less significant among $Ng^{+/+}$ subjects. These findings suggest that the wild-type mice contain supra-threshold levels of Ng for proficient performance of water maze tasks. $Ng^{-/-}$ mice performed poorly in all spatial tasks for which they were tested and exhibited deficits in LTP and concurrent CaMKII autophosphorylation. The tetanus-frequency response curve of $Ng^{-/-}$ mice is shifted to the right compared with that of the $Ng^{+/+}$ mice; low-frequency stimulation (5–10 Hz) induced LTD in the former and modest LTP in the latter. Thus, Ng might regulate neuronal function by increasing the efficiency of $Ca^{2+}$- and $Ca^{2+}$/CaM-mediated signalling and thus improve the performance in behavioral tests.

### Role of NMDA-Mediated Phosphorylation and Oxidation of Neurogranin in $Ca^{2+}$- and $Ca^{2+}$/CaM-Regulated Neuronal Signaling in the Hippocampus

*Wu, Huang K, Huang F*

Activation of the NMDA receptor is one of the critical steps leading to the enhancement of synaptic plasticity, which underlies learning and memory. Calcium influx induced by activation of the receptor triggers the stimulation of PKC, $Ca^{2+}$/CaM-dependent protein kinases, adenylyl cyclases, and nitric oxide synthase. It is the activation of these $Ca^{2+}$- and $Ca^{2+}$/CaM-dependent enzymes and their downstream targets that contribute to NMDA receptor–dependent LTP. Stimulation of PKC causes phosphorylation of Ng, which promotes the release of $Ca^{2+}$ from intracellular stores through G protein–coupled phosphoinositide second messenger pathways. In addition, Ng is susceptible to oxidant-mediated modification, which causes glutathionylation and/or formation of intramolecular disulfide bonds. The latter modification, resembling PKC-mediated phosphorylation, also results in a reduction of binding affinity of CaM. Using mouse hippocampal slices, we showed that NMDA induced a rapid and transient phosphorylation and oxidation of Ng. NMDA also caused activation of various PKC isozymes as evidenced by their phosphorylation, notably at the carboxyl terminal autophosphorylation sites; such activations were much reduced in the Ng knock-out mice. A high degree of phosphorylation of $Ca^{2+}$/CaM-dependent kinase II and activation of cAMP-dependent protein kinase were also evident in the wild-type mice compared with the kinases of the knock-out mice. The findings demonstrate the functional role of Ng in $Ca^{2+}$- and $Ca^{2+}$/CaM-dependent signalling pathways subsequent to stimulation of the NMDA receptor, suggesting that both phosphorylation and oxidation of Ng may regulate neuronal signalling.

### Glutathionylation of Proteins by Glutathione Disulfide S-Oxide

*Huang K, Huang F*

Redox regulation through modifications of proteins has emerged as one of the major cellular responses to oxidative and nitrosative stresses. In the central nervous system, neurotransmission under normal or pathological conditions generates a variety of oxidants. A wide range of modifications of the sulfhydryl group in proteins, including formation of S-nitrosocysteine, cysteine sulfenic acid (Cys-SOH), sulfinic acid ($Cys\text{-}SO_2H$), sulfonic acid ($Cys\text{-}SO_3H$), inter- and intramolecular disulfide, and mixed disulfide with glutathione (GSH) (Cys-S-SG), have been identified. Many of these modifications are potential sensors of the redox state's response to changing environments that are induced by growth factors, hormones, neurotransmitters, and cytokines. Thionylation of protein is one of the mechanisms that can serve as protection against oxidative insults and can be involved in cell signalling. Resembling the well-characterized mechanism of protein phosphorylation in cellular regulation, protein S-glutathionylation adds ionic charges by introducing the $\gamma$-glutamyl tri-peptide into a protein. The potential target proteins for thionylation are likely to be as abundant as those for phosphorylation. However, unlike protein phosphorylation, for which numerous protein kinases have been identified, there is no evidence for the involvement of a specific thionylating enzyme for each target protein. Thus, the specificity for the thionylation may be endowed on each protein by its affinity for the modifiers, namely, thionylating agents, and the accessibility of the sulfhydryl group. The thionylating agent must be highly reactive and preferably have a short half-life so that the reaction will be localized near the origin of the oxidant. Recently, we identified a highly reactive glutathionylating agent, glutathione disulfide S-oxide (GS(O)SG) from the aqueous solution of S-nitrosoglutathione (GSNO), that fulfills some of these criteria. This compound, also called glutathione thiosulfinate, is the anhydride of glutathione sulfenic acid (GSOH). Introduction of an oxygen atom into a disulfide bond significantly decreases the bond energy and transforms it into a highly reactive agent toward thiol, resulting in the formation of mixed disulfides. The rate of reaction of GS(O)SG with 5-mercapto-2-nitrobenzoate was nearly 20-fold higher than that of GSNO. The mechanism for the formation of GS(O)SG is believed to involve the sulfenic acid (GSOH) and thiosulfinamide (GS(O)$NH_2$) intermediates; the former undergoes self-condensation and the latter reacts with GSH to form GS(O)SG. Many reactive oxygen and nitrogen species are also capable of oxidizing GSH or GSSG to form GS(O)SG, which probably plays a central role in integrating the oxidative and nitrosative cellular responses through thionylation of thiols. Treatment of rat brain tissue slices with oxidants resulted in enhanced thionylation of proteins with a concomitant increase in the cellular level of GS(O)SG, suggesting that the compound may play a second messenger role for stimuli that produce a variety of oxidative species.

## SELECTED PUBLICATIONS

1. Huang KP, Huang FL. Glutathionylation of proteins by glutathione disulfide S-oxide. Biochem Pharmacol. 2002; 64:1049-1056.
2. Li J, Huang FL, Huang KP. Glutathiolation of proteins by glutathione disulfide S-oxide derived from S-nitrosoglutathione. Modifications of rat brain neurogranin/RC3 and neuromodulin/GAP-43. J Biol Chem. 2001;276:3098-3105.
3. Miyakawa T, Edom Y, Pak JH, Huang FL, Huang KP, Crawley JN. Neurogranin null mutant mice display performance deficits on spatial learning tasks with anxiety related components. Hippocampus. 2001;11:763-775.
4. Watson JB, Khorasani H, Persson A, Huang KP, Huang FL, O'Dell TJ. Age-related deficits in long-term potentiation are insensitive to hydrogen peroxide: coincidence with enhanced autophosphorylation of $Ca^{2+}$/calmodulin-dependent protein kinase II. J Neurosci Res. 2002;70:298-308.
5. Wu J, Li J, Huang KP, Huang FL. Attenuation of protein kinase C and cAMP-dependent protein kinase signal transduction in the neurogranin knockout mice. J Biol Chem. 2002;277:19498-19505.

## COLLABORATORS

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# THE BIOTRANSFORMATION OF ENDOBIOTICS BY SULFONATION

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Our overall research challenge is to gain insight into the molecular mechanisms and biologic significance of the modification of substances by sulfonation, a fundamental process in the biotransformation of endobiotics as well as of drugs and xenobiotics. Sulfonation is essential for normal growth and development and maintenance of the internal milieu. Sulfonated macromolecules such as glycosaminoglycans and proteoglycans are involved in cell surface and connective tissue structures and bone formation. Sulfonation of tyrosine residues is a widespread post-translational modification of many secretory and membrane proteins. In addition, glycoprotein hormones are modified by the sulfonation of specific saccharide moieties that create unique structural motifs. Sulfolipids are concentrated in the brain, peripheral nerves, and reproductive tissues. Sulfonation is also of major importance in the biotransformation of low molecular weight compounds such as catecholamines, iodothyronines, neuroendocrine peptides, and cholesterol and its metabolites (oxysterols, bile acids, vitamin D, and steroid hormones). By modulating availability of the biologically active form, sulfonation markedly influences the biologic activity of steroids/sterols. Thus, sulfotransferases play an essential role in specific physiologic systems and their associated disorders, e.g., the menstrual cycle, sperm capacitation and fertility, hormone-dependent tumors of prostate and breast, obesity and diabetes, lung maturation, and respiratory distress syndrome.

### PAPS Synthase Characterization and Expression

*Fuda, Lee, Shimizu, Strott*

Sulfonation cannot occur in the absence of the universal sulfonate donor molecule 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The production of PAPS from ATP and inorganic sulfate is carried out by a bifunctional enzyme, the product of two separate genes, i.e., PAPS synthase 1 and PAPS synthase 2. In addition, as a result of differential splicing, PAPS synthase 2 occurs as two subtypes, creating a total of three PAPS synthase isoforms. We have cloned and characterized both gene products in humans as well as in guinea pigs and rabbits. It is not clear why two genes regulate PAPS formation; furthermore, the biochemical and physiological relationships of the PAPS synthase isoforms remain poorly understood. We have examined differences in the genes' amino acid composition and whether the differences in composition might be associated with functional differences. All three isoforms demonstrate Michaelis/Menten kinetics, but the PAPS synthase 2 isoforms have a greater catalytic efficiency ($k_{cat}/K_m$) and are 15 to 20 times more active than PAPS synthase 1. Multiplex RT-PCR reveals that PAPS synthase1 is expressed in essentially all human tissues, suggesting that the gene is constitutively expressed. In contrast, PAPS synthase 2 is expressed in a tissue-specific manner; moreover, the PAPS synthase 2 subtypes are differentially expressed, suggesting tissue-specific regulation of the gene as well as differential splicing.

Human PAPS synthase 2 was discovered during a search for the genetic basis of a developmental abnormality causing a form of spondyloepimetaphyseal dysplasia that presents with a skeletal phenotype involving the spine and long bones. Initially, it was baffling that in the genetic disorder involving human PAPS synthase 2, which produces the osteochondrodysplasia phenotype, the cartilage-specific defect occurs despite the co-expression of PAPS synthase 1 in cartilaginous tissue. In fact, as we have been able to demonstrate, PAPS synthase 1 is more vigorously expressed than PAPS synthase 2 in cartilage from human adults. The pathology in human spondyloepimetaphyseal dysplasia is attributable to undersulfation of extracellular matrix proteoglycans. We believe that this apparent enigma has been resolved by demonstrating that, in the cartilaginous growth plate of guinea pig long bones, PAPS synthase 2 is indeed the predominantly expressed isoform, whereas PAPS synthase 1 is poorly expressed. Such appears to be a general phenomenon in immature animals, i.e., cartilage obtained from non-growth plate sites (e.g., sternum and rib cage) also more vigorously expresses PAPS synthase 2 in contrast to the situation with mature animals, which, as the human adults, more prominently express PAPS synthase 1 in their cartilage. It is highly likely that this finding involving immature guinea pigs' cartilage as an animal model will also hold true for human development where tissues are not readily available for examination.

### PAPS Synthase Transcriptional Regulation

*Shimizu, Fuda, Lee, Strott*

The gene for PAPS synthase 1 contains neither a TATAAA nor a CCAAT box. The RNA ligase–mediated rapid amplification of cDNA ends (RLM-RACE) method, a recently developed variation of the 5'RACE procedure, revealed the presence of two possible transcription start sites, as confirmed by RT-PCR experiments that used gene-specific primers. We determined the proximal promoter region and identified multiple GC/GT boxes. Two of the elements upstream of the proximal promoter region and two downstream GC/GT boxes nearest the transcription start site significantly influence promoter activity. Nuclear extracts of SW13 cells, which highly express PAPS synthase 1, contain proteins that bind to probes possessing specific GC/GT boxes. The gene for PAPS synthase 1 appears to be under the influence of the Sp1 family of transcription factors, i.e., supershift analysis confirmed the presence of Sp1, Sp2, and Sp3 proteins in the nuclear extract. Co-transfection experiments using SL2 cells yielded additional support for the involvement of Sp1 in transcriptional regulation of the PAPS synthase 1 gene.

As with the gene for PAPS synthase 1, the promoter for PAPS synthase 2 contains neither a TATAAA nor a CCAAT box, although a consensus initiator motif is present downstream of the cap site. We determined two transcription initiation sites similar to PAPS synthase 1 for PAPS synthase 2. We confirmed the putative start sites by RT-PCR experiments that used gene-specific primers. The proximal promoter for HepG2, JEG3, and SW13 cell lines is similarly located between bp -84 and bp -124 upstream of the putative transcription start site. The region contains two GC/GT boxes that are essential for full promoter activity, as indicated by deletion analysis and further supported by mutagenesis. A nuclear extract of SW13 cells, which highly express PAPS synthase 2 as well as PAPS synthase 1, contains proteins that bind to probes possessing promoter-specific GC/GT boxes; furthermore, similar to the case with PAPS synthase 1, supershift analysis confirmed the presence of Sp1, Sp2, and Sp3 proteins in the nuclear extract, while co-transfection experiments using SL2 cells yielded additional support for involvement of Sp1 in transcriptional regulation of the PAPS synthase 2 gene.

### Kinetic Analysis and Differential Expression of Human Hydroxysteroid Sulfotransferase SULT2B1 Isoforms

*Fuda, Lee, Higashi, Javitt, Strott*

Due to the presence of an alternative exon 1, the human hydroxysteroid sulfotransferase gene *SULT2B1* encodes two subtypes that structurally differ only at their amino termini. The influence of the unique amino termini on substrate specificity, however, has remained unclear. Interestingly, kinetic data demonstrate that the SULT2B1 isoforms and the prototypical SULT2A1 isozyme exhibit distinct substrate preferences. For instance, SULT2B1a avidly sulfonates pregnenolone but not cholesterol, whereas SULT2B1b selectively sulfonates cholesterol, suggesting that the latter isoform is the true human cholesterol sulfotransferase. Notably, in contrast to SULT2A1, which is regarded as a dehydroepiandrosterone (DHEA) sulfotransferase, neither SULT2B1 isoform efficiently sulfonates DHEA. These distinct substrate predilections, along with their differential expression patterns, strongly suggest that the SULT2 isozymes have distinct roles. Cholesterol sulfate is widely distributed and, in human plasma, is the predominant steroid sulfate. Because sulfonation of cholesterol is crucial to keratinocyte differentiation as well as to the development of the barrier and thus to normal skin development, the finding that SULT2B1b functions as a cholesterol sulfotransferase and is the predominant steroid/sterol sulfotransferase expressed in human skin has proved significant. In contrast, the prototypical steroid sulfotransferase SULT2A1 does not sulfonate cholesterol and is not expressed in human skin.

## Structure/Function Analysis of the Human Hydroxysteroid Sulfotransferase SULT2B1 Isoforms

*Fuda, Shimizu, Lee, Javitt, Strott*

From a structural point of view, compared with the SULT2A1 isozyme, the outstanding feature of the SULT2B1 isoforms as well as other of cloned steroid and cognate cytosolic sulfotransferases is their extended amino- and carboxy-terminal ends. All previously cloned members of the mammalian cytosolic sulfotransferase superfamily, i.e., estrogen and phenol sulfotransferases as well as hydroxysteroid sulfotransferases, are composed of 282 to 295 amino acids, whereas SULT2B1a and SULT2B1b consist of 350 and 365 amino acids, respectively. Nonetheless, the SULT2A1 and SULT2B1 isozymes exhibit a significant structural similarity in their core regions, most notably among the amino acid residues important in protein-PAPS co-factor interaction of cytosolic sulfotransferases, which are completely conserved.

The functional significance of the extended carboxyterminal end of the SULT2B1 isoforms is not known. One speculation is that this region, which is enriched in prolines, may play a role in protein-protein interactions. It is notable that the relatively long carboxyterminal extension (53 amino acids) common to both SULT2B1 isoforms can be removed without causing a significant change in the catalytic behavior of either isoform. On the other hand, removal of the unique amino-terminal ends, which distinguish the SULT2B1 isoforms, produces interesting results. Removal of the unique 23 amino-terminal residues of SULTT2B1b abolishes catalytic activity, whereas removal of the unique eight amino-terminal residues of SULT2B1a does not; indeed, the first 18 amino acids of the SULT2B1b amino-terminal region are not essential for catalytic activity, although the five-amino acid segment between residues 18 and 24 from the amino terminus of SULT2B1b is essential for catalytic activity. Within this five-amino acid DISEI sequence of SULT2B1b, it is the isoleucines that are critical for catalysis. Moreover, amino acid substitutions show that only a hydrophobic amino acid with a side chain of optimal size can function effectively at these positions, i.e., alanine cannot fulfill this function, whereas leucine and isoleucine work equally well; furthermore, methionine, although less effective than isoleucine, is also able to sustain significant enzymatic activity. Conversely, charged and polar amino acid substitutions at either of the isoleucine positions are inactive.

The human *SULT2B1* gene consists of an exon 1B, an exon 1A, and exons 2 through 6: the SULT2B1a isoform is encoded by exons 1A and 2 through 6; the SULT2B1b isoform is encoded by exon 1B, the final 143 nucleotides of exon 1A plus exons 2 through 6. Exon 1B is composed of a 5'UTR consisting of 129 nucleotides and the coding region for the first 23 amino acids of SULT2B1b, which represents the entire amino-terminal region that is unique to this isoform. Exon 1A, on the other hand, in addition to a 179-nucleotide 5'UTR, encodes the first 56 amino acids of SULT2B1a, of which only the first eight amino acids are unique to this isoform. Thus, when exon 1B of the human *SULT2B1* gene is transcribed, cholesterol sulfotransferase is synthesized, whereas when exon 1A is transcribed, pregnenolone sulfotransferase is produced.

For several reasons, the most suitable organ systems in which to consider the physiological implications of the differential expression of the human *SULT2B1* gene are skin and brain. First, cholesterol sulfate plays an essential role in skin development and expression of the human SULT2B1b isoform, which is now recognized as a cholesterol sulfotransferase. Second, cholesterol sulfate levels are highest in skin. Third, the human fetal brain, as determined by RT-PCR, expresses only the SULT2B1a isoform. Finally, sulfated pregnenolone, which is produced most efficiently by the action of the SULT2B1a isoform, is now appreciated as an essential neurosteroid.

### Mouse Ortholog of Human SULT2B1

*Shimizu, Fuda, Yanei, Strott*

We have cloned a mouse SULT2B1 cDNA (designated SULT2B1a) and found that it is homologous to a previously cloned mouse cDNA (designated SULT2B1b). The mouse *SULT2B1* gene structure is identical to that of the human *SULT2B1* gene, indicating that the gene is highly conserved. Thus, similar to the human SULT2B1 isoforms, the mouse counterparts arise from a single *SULT2B1* gene by using an alternative exon 1 coupled with differential splicing. Therefore, as with the human SULT2B1 isoforms, the mouse SULT2B1 isoforms differ only at their amino termini. Furthermore, the mouse SULT2B1 isoforms, like human SULT2B1, have a predilection for cholesterol over DHEA, which is ordinarily sulfonated by the mouse SULT2A1 subfamily of hydroxysteroid sulfotransferases.

The differential expression of mouse SULT2B1a, SULT2B1b, and SULT2A1, along with their distinct substrate preferences, no doubt reflects their physiologic roles. Thus, the exclusive expression of SULT2B1a in the central nervous system and its preference for pregnenolone as a substrate are in keeping with the importance of pregnenolone sulfate as a neurosteroid; the exclusive expression of SULT2B1b in skin and its preference for cholesterol as a substrate are in keeping with the importance of cholesterol sulfate as a regulatory molecule in keratinocyte differentiation and development of the epidermal barrier; and the exclusive expression of SULT2A1 in the liver is in keeping with its role in general metabolism involving xenobiotics as well as endobiotics. Interestingly, the mouse *SULT2B1* and *SULT2A1* genes are differentially expressed during embryonic development, the former at all stages from E8.5 through E19 and the latter not until E19. The biologic and developmental significance of this observation remains to be elucidated. To this end, we aim to develop a gene knock-out mouse model, first disrupting the gene for SULT2B1 and then constructing double knock-out of the SULT2B1 and SULT2A1 genes.

## SELECTED PUBLICATIONS

1. Fuda H, Lee YC, Shimizu C, Javitt N, Strott CA. Mutational analysis of human hydroxysteroid sulfotransferase SULT2B1 isoforms reveals that exon IB of the SULT2B1 gene produces cholesterol sulfotransferase, whereas exon IA yields pregnenolone sulfotransferase. J Biol Chem. 2002;277:36161-36166.
2. Fuda H, Shimizu C, Lee YC, Akita H, Strott CA. Characterization and expression of human 3'-phosphoadenosine 5-phosphosulfate synthase isoforms. Biochem J. 2002;365:497-504.
3. Shimizu C, Fuda H, Lee YC, Strott CA. Transcriptional regulation of human 3'-phosphoadenosine 5'-phosphosulfate synthase 2. Biochem J. 2002;363:263-271.
4. Strott CA. Dehydroepiandrosterone and cholesterol sulfotransferases. In: Coughtrie MWH, Pacifici GM, eds. Human cytosolic sulfotransferases. London;Taylor & Francis, 2002;in press.
5. Strott CA. Sulfonation and molecular action. Endocr Rev. 2002;23:703-732.

# CONNECTIVE TISSUE DISORDERS

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In a unique integrated program of laboratory and clinical investigation, we study the molecular biology of the heritable connective tissue disorders osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS). Our objective is to elucidate the mechanisms by which primary collagen defects cause skeletal fragility and other significant connective tissue symptoms and then to apply the knowledge gained from our studies to the treatment of children with these conditions. An understanding of the interactions of mutant collagen molecules with the normal collagenous and non-collagenous components of extracellular matrix will also enhance our understanding of normal bone function and may yield insights applicable to the more common forms of osteoporosis. We recently focused on the development of a non-lethal animal model for OI with a classical collagen mutation. This non-lethal knock-in mouse, the Brtl mouse (Brtl), with a glycine substitution mutation in the $\alpha1(I)$ chain, is an excellent model for pharmacological treatment trials, for approaches to gene therapy suitable for dominant disorders, and for investigations of the skeletal matrix of OI. Our clinical studies involve children with types III and IV OI who are enrolled in age-appropriate clinical protocols for treatment and form a longitudinal study group.

## The Brtl Mouse Model for OI

*Marini, Uveges, Moriarty, Zhao; in collaboration with Raggio, Forlino, Goldstein*

We have generated a knock-in murine model for OI that carries a classical OI mutation in type I collagen under the control of the endogenous promoter; we named the model the Brittle (Brtl) mouse. We introduced a point mutation into one murine *col1a1* allele causing a gly349cys substitution that was modeled on the collagen defect present in one of our type IV OI patients. We selected it for duplication in the mouse because it was non-lethal in humans and was typical of the glycine substitutions in type I collagen that cause about 85 percent of OI cases.

We have investigated the skeletal adaptation of the Brtl mouse during puberty by using a combination of bone density measurements (BDM), biomechanics, and histomorphometry. It is well known that fractures in type IV OI decrease after puberty, but the mechanism of the change remains unknown. BDM of the Brtl femur and spine is about 70 percent of wild type before puberty. After puberty, the BMD of the wild-type mice does not change significantly while that of the post-pubertal Brtl mouse is significantly greater than the pre-pubertal Brtl, attaining 90 percent of wild-type BMD. The change in the amount, composition, or organization of the mineral phase of the skeleton during puberty in the Brtl mouse is distinct from pubertal development in the wild-type mouse. The increased strength of the Brtl bone in the context of weak geometry points to changes in the composition of the Brtl bone material itself as the key to post-pubertal adaptation. Understanding the physiological processes that cause improved bone strength and the mechanisms that control them may provide novel approaches to the therapy of OI.

In collaboration with investigators at the Hospital for Special Surgery, we are comparing the effects of bisphosphonate alendronate on the Brtl mouse with those on a recessive mouse model for OI, the oim mouse. The treatment of Brtl mice with alendronate is complementary to our patient treatment trial with bisphosphonate. The murine study will provide skeletal material for biomechanics, histology, and matrix composition investigations. For example, the inhibition of bone remodeling by the bisphosphonate drugs might cause increased brittleness of OI bone, a potential side effect that cannot be studied in patients. Our preliminary data indicate that bisphosphonate treatment does not alter the growth patterns of either Brtl or wild-type mice, with the treated Brtl remaining about 60 percent of the wild-type size through puberty.

### Ribozyme Approach to OI Gene Therapy

*Uveges, Marini*

We have taken a mutation suppression approach to gene therapy of the dominant negative connective tissue disorders. Suppression of the level of mutant collagen transcripts would, in principle, transform a structural collagen mutation with severe clinical consequences into a quantitative mutation with mild to undetectable clinical symptoms. We are using hammerhead ribozymes as the mutation suppression agent. Previously, we demonstrated the specificity and effectiveness of hammerhead ribozymes *in vitro* and in cultured OI cells. We are currently engaged in generating transgenic mice with the ribozyme under *tet* control to achieve high levels of ribozyme expression. The mice will be bred to the Brtl mice for investigation of the effect of ribozyme on collagen mutations and the OI phenotype *in vivo.*

### Parental Mosaicism as a Model for OI Cell Therapy

*Cabral, Marini*

Parents who are mosaics for the collagen mutations that cause clinically significant OI in their children are models for the cell therapy approach to OI. Although these individuals carry the collagen mutation in a fraction of their cells, they are clinically normal or minimally affected. In cell therapy, a portion of the osteocytes in an affected individual would be replaced with normal cells, resulting in a mosaic individual who might have improved skeletal function. One major deficit in the scientific rationale for cell therapy has been a lack of information about the proportion of heterozygous bone cells present in genetic mosaic individuals. We have examined skeletal cells from two asymptomatic mosaic carriers with COL1A1 mutations. Each mosaic carrier has a high proportion of dermal fibroblasts that are heterozygous for the collagen defect that causes OI in their children. Carriers for types IV OI and III OI were studied by using both labeled and single-cell PCR. Despite a 45 to 75 percent burden of mutant cells, both women had normal bone growth, density, and histology and minimal clinical signs. Thus, our data provide the first demonstration that a significant burden of mutant osteoblasts is compatible with normal skeletal functioning, allowing us to set the goal for cell therapy of OI at tentatively 50 to 60 percent normal cells.

### Study of Collagen Mutations Causing OI

*Cabral, Masella, Moriarty, Marini; in collaboration with Leikin*

We have been investigating the consequences of a rare type of collagen mutation on collagen assembly, stability, and incorporation into fibrils and matrix. We delineated a triplet duplication in COL1A1 exon 44; the normal allele encodes three identical Gly-Ala-Hyp triplets while the mutant allele encodes four. This mutation shifts the register of the collagen chains with respect to each other but does not interrupt the triplet sequence, and yet it causes a lethal phenotype. The realignment of X and Y positions caused by the register shift delays helix formation, causing overmodification. Differential scanning calorimetry yields 2°C decreased stability of helices with one mutant chain and 6°C decreased stability of helices with two mutant chains. The register shift persists throughout the entire helix and decreases the rate of N-proteinase processing. The shift also disrupts incorporation of mutant collagen into fibrils and matrix. Collagen helices with two mutant chains and a significant portion of helices with one mutant α1(I) chain do not participate in fibril formation. This exclusion of mutant chains would be expected to cause dramatically decreased matrix production *in vivo*. In matrix deposited by proband fibroblasts in culture, mutant chains were well incorporated into the immaturely cross-linked fraction but constituted a minor fraction of maturely cross-linked chains. The profound biochemical effects of shifting the collagen register correlate with the severe clinical consequences. Given that double mutant molecules were most severely impaired in stability and fibrillogenesis, alignment of the α1(I) chains with the α2(I) X and Y positions appears to be critical.

Other investigations involve collagen mutations at the amino end of the helical region of α1(I) collagen. These mutations do not result in collagen overmodification but do cause destabilization of the helix as detected by differential scanning calorimetry. In addition, at the opposite end of pro-α1(I), an NIH OI patient with type IV OI has a notable collagen mutation located in the carboxy-terminal propeptide. Since the mutation itself would not be present in the mature collagen that is incorporated into helix, it is interesting that the patient has significant bone disease. We are examining the biochemical and matrix formation consequences of this mutation. Finally, we are investigating possible modifying factors for OI. In particular, we are exploring several polymorphisms that are known to be associated with post-menopausal osteoporosis.

### Treatment of Children with OI with Bisphosphonate and/or Growth Hormone

*Letocha, Marini*

We have been conducting a four-arm treatment trial of the bisphosphonate pamidronate and recombinant growth hormone (rGH). Children with types III and IV OI are randomized among four groups: pamidronate alone, rGH alone, both drugs, or no drugs. Given that growth hormone stimulates osteoblasts to produce bone matrix and that the bisphosphonate inhibits resorption by osteoclasts, the two drugs could act synergistically to increase quantities of bone matrix.

In the wake of uncontrolled pilot studies, the OI community has voiced strong demand for bisphosphonate administration. Our study, however, is the only controlled trial of pamidronate in OI children. Major endpoints include lumbar spine bone density and vertebral compressions. Because it is possible that increased quantities of bone matrix containing abnormal type I collagen might lead to increased brittleness of bone, we will devote special effort to determining whether the quality of the bone matrix is improved. Other protocols investigate the natural history of basilar invagination in OI and the incidence and progression of pulmonary and cardiac complications.

## SELECTED PUBLICATIONS

1. Bouma P, Cabral WA, Cole WG, Marini JC. Col5A1 exon 14 splice acceptor site mutation causes a functional null allele and abnormal heterotypic interstitial fibrils in EDS II. J Biol Chem. 2001;276:13356-13364.
2. Cabral WA, Chernoff EJ, Marini JC. G76E substitution in type I collagen is the first non-lethal glutamic acid substitution in the a1(I) chain and alters folding of the N-terminal end of the helix. Mol Genet Metab. 2001;72:326-335.
3. Cabral WA, Fertala A, Green LK, Korkko J, Forlino A, Marini JC. Procollagen with skipping of a1(I) exon 41 has lower binding affinity for α1(I) C-telopeptide, impaired in vitro fibrillogenesis and altered fibril morphology. J Biol Chem. 2002;277:4215-4222.
4. Chernoff E, Marini JC. Osteogenesis imperfecta. In: Allanson J, Cassidy S, eds. Clinical management of common genetic syndromes. New York: Wiley & Sons, 2001;Chapter 17:281-300.
5. Marini JC. Genetic risk factors for lumbar disk disease. Invited editorial. JAMA. 2001;285:1886-1887.
6. Marini JC. Heritable collagen disorders. In: Hochberg M et al., eds. Rheumatology, 3rd ed., Chapter 44. London: WB Saunders, Mosby, Churchill, Livingston; in press.

## COLLABORATORS

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# MOLECULAR BIOLOGY, REGULATION, AND BIOCHEMISTRY OF UDP-GLUCURONOSYLTRANSFERASE ISOZYMES

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Mammalian UDP-glucuronosyltransferase (UGT) isozymes are critical catalysts in the broad and vital function of detoxifying endogenous products and potentially injurious lipid-soluble phenols derived from the diet and the environment. The isozymes maintain chemical homeostasis and detoxify by conjugating metabolites, drugs, toxins, and environmental chemicals with glucuronic acid, yielding water-soluble excretable products. The system prevents accumulation of lethal plasma levels of bilirubin, inactivates many drugs, and averts mutagenicity and carcinogenicity of polycyclic aromatic hydrocarbons, including benzo(a)pyrene, which is found in cigarette smoke and automobile emissions. Moreover, UGT prevents accumulation of high concentrations of dietary-derived polyphenols that inhibit critical enzymes. An important research aim is to understand the specific, still unknown properties and enzymatic mechanism(s) that allow UGT to convert numerous structurally unrelated lipid-soluble phenols to innocuous glucuronides.

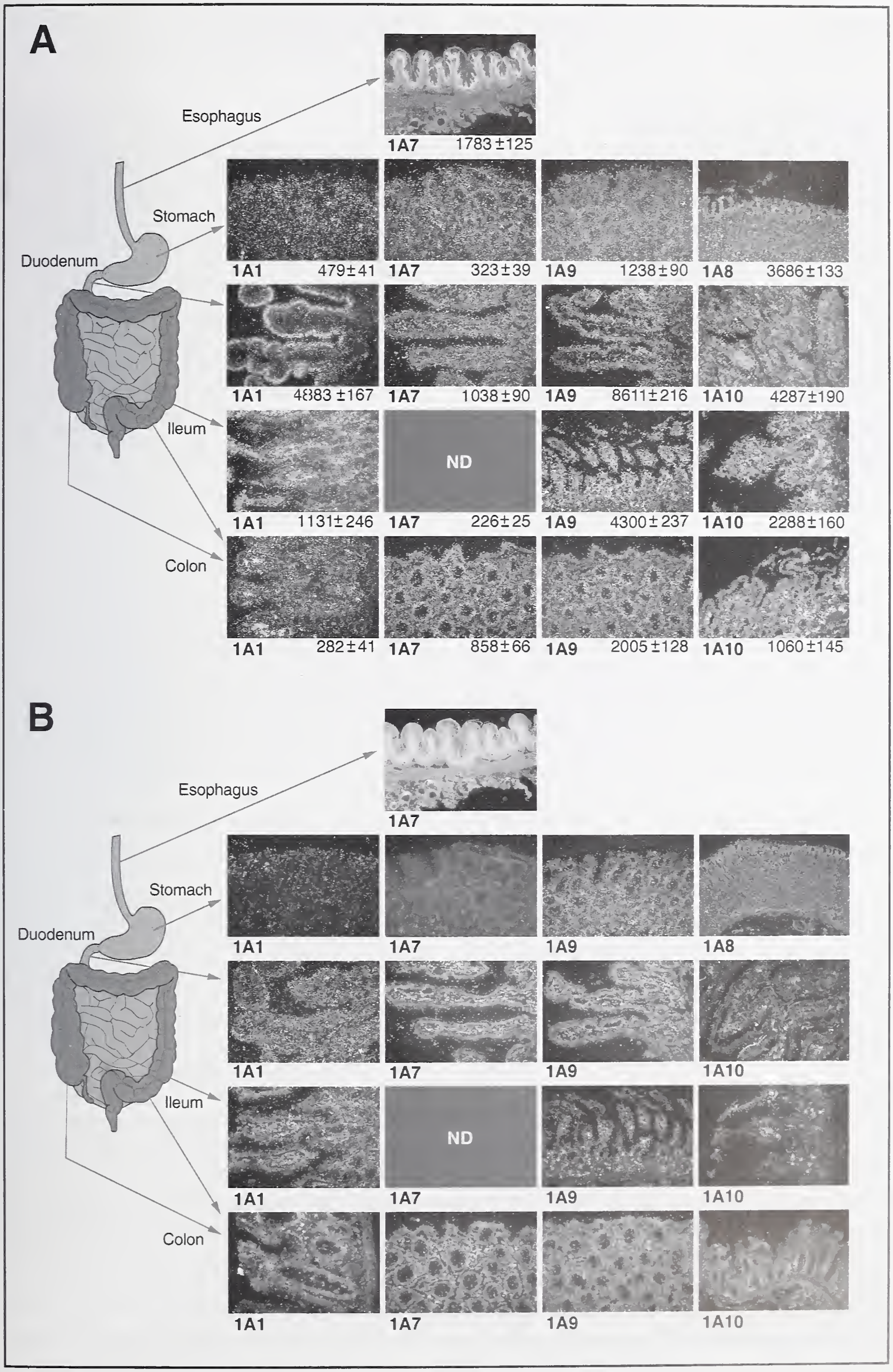
## Phosphorylation of UGT Proteins with Signalling

*Basu, Kole, Kubota, Mitra, Pennington*

While attempting to understand the glucuronidation requirements of UGTS by using human colon cell lines, we discovered that the isozymes require phosphorylation. Phosphorylation is mediated by at least two protein kinase C (PKC) isozymes while the action of classical PKC agonists and antagonists, as well as the effects of PKC translocation-specific inhibitor peptides, confirmed the involvement of signalling events. Mutants for two predicted phosphorylation sites in UGT were dominant negative, and a third site indicated that the phospho-group(s) plays a unique and novel role in catalysis by controlling pH optima and substrate selection. Expression of UGT activity and its mutants, in conjunction with over-expression of PKCs, demonstrated site-specific phosphorylation. Evidence from 10 different UGTs indicates that phosphorylation is a broadly based requirement. Hydrogen peroxide stimulation of constitutive UGT and inhibition of $H_2O_2$-enhanced activity by catalase and herbimycin

**FIGURE 12 (right)**

Cellular localization of mRNA encoding UGT1A1 and UGT1A7-10. *In situ* hybridization with antisense probes (A) and sense probes (B). The esophageal images were magnified 50x; all others were magnified 200x. Numbers below each panel represent glucuronidation by microsomes isolated from adjacent tissue in a two-hour incubation (pmol/hr/gm tissue) for comparison with the RNA levels depicted by *in situ* analysis. The substrates were bilirubin, thymol, phloretin, apigenin, and epigallocatechin 3-0-gallate for UGT1A1 (pH 6.4), UGT1A7 (pH 7.6), UGT1A8 (pH 7.0), UGT1A9 (pH 6.4), and UGT1A10 (pH 6.4), respectively. Substrates are 95 percent specific, except thymol and phloretin, which were 60 and 33 percent specific for UGT1A7 and UGT1A8, respectively. ND: not detectable.


A
Esophagus
Stomach
Duodenum
Ileum
Colon
1A7 1783±125
1A1 479±41
1A7 323±39
1A9 1238±90
1A8 3686±133
1A1 4883±167
1A7 1038±90
1A9 8611±216
1A10 4287±190
1A1 1131±246
1A7 226±25
ND
1A9 4300±237
1A10 2288±160
1A1 282±41
1A7 858±66
1A9 2005±128
1A10 1060±145
B
Esophagus
Stomach
Duodenum
Ileum
Colon
1A7
1A1
1A7
1A9
1A8
1A1
1A7
1A9
1A10
1A1
1A7
ND
1A9
1A10
1A1
1A7
1A9
1A10

suggest that cellular oxidants are signal(s) for the PKC-UGT system. Our results demonstrate a linkage between oxidative stress–stimulated phosphorylation of PKC and the critical cellular function of detoxification.

### Effect of Location and Properties of UGT in the Control of Gastrointestinal Chemical Absorption

*Basu, Kole; in collaboration with McDonagh*

Using Northern blot, *in-situ*, and biochemical analyses, we determined distribution and function of the bilirubin-metabolizing isozyme UGT1A1 and of the UGT1A7-10 isozymes. We found, for the first time, that the isozymes are segmentally distributed throughout the gastrointestinal (GI) mucosal epithelia (Fig. 12A and B). Recombinant isozymes exhibited pH optima of 6.4 or 7.6 and/or broad ranges; increasing substrate concentrations either did not effect or progressively inhibited activity. Under different optimal conditions, all isozymes exhibited wide substrate specificity for diet- and environmentally associated chemicals. We demonstrated the impact of glucuronidation on drug and chemical absorption at the GI level by exploiting our recent finding that UGTs require phosphorylation. [$^{33}$P]Orthophosphate incorporation into immunoprecipitable UGTs demonstrated phosphorylation. The simultaneous loss of UGT radiolabeling and activity in cell lines by the general kinase inhibitor curcumin and the PKC-specific inhibitor calphostin-C confirmed the requirement for phosphorylation. Using curcumin-treated mouse duodenal loops, we demonstrated that free bilirubin (a marker) uptake dramatically increased while the levels of bilirubin-glucuronides in portal blood, lumen, and tissue declined. Our results represent the first direct evidence that UGTs control GI absorption of polyphenols and that maintenance of their phosphorylation is required to limit chemical absorption.

### Immunosuppressant Activity of Mycophenolic Acid Improved by Transient Inhibition of Gastrointestinal UGT

*Basu, Kole*

In view of extensive glucuronidation and resulting high dosage requirements, the promising immunosuppressant mycophenolic acid (MPA), used now in transplantation and autoimmune disease protocols, exhibits serious side effects. We found that the cellular distribution and biochemical properties of UGT1A7-10, primary metabolizers of MPA, contribute significantly to high oral-dose requirements. *In situ* hybridization studies revealed that UGT1A7-, UGT1A9-, and UGT1A10-mRNAs are located in GI mucosal epithelia; studies with microsomes isolated from adjacent specimens showed that esophagus, ileum, duodenum, and colon have high to moderately high glucuronidating activities. Recombinant UGTs avidly glucuronidate MPA, showing nearly linear increases in activity at concentrations as high as 800 µM; only UGT1A9 showed typical saturation kinetics. Each isozyme generates about 80 percent ether-linked and about 20 percent acylglucuronide. To establish the *in vivo* impact of MPA glucuronidation, we treated LS189 colon cells with the general kinase inhibitor curcumin and the highly specific protein kinase C inhibitor calphostin-C to inhibit UGTs, targeting the newly discovered phosphorylation requirement. Concentration-dependent inhibition of [$^{33}$P]orthophosphate labeling of UGTs by calphostin-C, with parallel loss of activity, confirmed the UGT's phosphorylation. Transient inhibition of glucuronidation by oral pretreatment with curcumin before MPA administration caused nearly a three-fold greater immunosuppression of antigen-stimulated spleen cytotoxic T-lymphocyte proliferation in mice. Hence, curcumin-pretreatment to inhibit GI-distributed UGT is a potential model for increasing the bioavailability of highly glucuronidated drugs.

## SELECTED PUBLICATIONS

1. Gong QH, Cho JW, Huang T, Potter C, Gholami N, Basu NK, Kubota S, Carvalho S, Pennington MW, Owens IS, Popescu NC. Thirteen UDP-glucuronosyltransferase genes are encoded at the human UGT1 gene complex locus. Pharmacogenetics. 2001;11:357-368.
2. Sugatani J, Kojima H, Ueda A, Kakizaki S, Yoshinari K, Gong QH, Owens IS, Negishi M, Sueyoshi T. The phenobarbital response enchancer module in human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. Hepatology. 2001;33:1232-1238.

## COLLABORATORS

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# INBORN ERRORS OF METABOLISM

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We investigate rare and informative inborn errors of metabolism, including Hermansky-Pudlak syndrome, cystinosis, and alkaptonuria. These rare diseases offer insight into biochemical and cellular mechanisms. Both gene discovery and mutation analysis assist in elucidating normal pathways and functions as well as provide a measure of genotype-phenotype correlation. In addition, we care for affected patients, providing expertise in largely abandoned diseases and thereby improving diagnosis, offering prognosis, and developing therapies for disorders that have none. Members of the section are considered the world's experts in cystinosis, Hermansky-Pudlak syndrome, and disorders of free sialic acid metabolism.

### Hermansky-Pudlak Syndrome

*Gahl, Huizing, Anikster, Anderson, Claassen, Bernardini; in collaboration with Boissy, White, Brantly, Gochuico, Avila*

We continue to discover mutations in the four known genes causing Hermansky-Pudlak syndrome (HPS) and to search for new genes responsible for the disease. We have described the murine gene corresponding to the human *HPS3* gene. In collaboration with other scientists, we have developed an assay to detect serotonin in platelets, evaluated a clotting analyzer for humans with platelet defects, and delineated the radiographic features of HPS patients with pulmonary fibrosis. We have examined, cared for, and investigated 120 HPS patients to date. In separate studies, we found that the anti-inflammatory drug, pirfenidone, helped retard the pulmonary fibrosis of HPS in patients who initially had at least 50 percent of the expected forced vital capacity.

### Cystinosis

*Gahl, Bernardini, Kleta, Lynch, Anikster, Phornphutkul; in collaboration with Thoene, Schneider, Kaiser-Kupfer, Skovby*

We follow approximately 60 patients with cystinosis, some before and some after renal transplantation. We perform mutation analysis on patients new to the protocol, accumulate longitudinal data on treatment regimens, and detect early and late complications of cystinosis. We have described the *CTNS* mutations identified in African American patients and, in collaboration with Danish investigators, in Danish patients. In collaboration with physicians in the National Eye Institute, we have helped to describe anterior segment complications of cystinosis and retinal visualization in cystinosis by using indocyanine green videoangiography. We also conducted a study of a new formulation of cysteamine eyedrops. Members of the section are helping to bring cysteamine eyedrops to New Drug Approval by the Food and Drug Administration.

### Alkaptonuria

*Gahl, Phornphutkul, Bernardini*

Alkaptonuria results from the accumulation of homogentisic acid, an intermediate in tyrosine degradation that results from a deficiency of homogentisate 1,2-dioxygenase. The symptoms include joint and cardiac valve deterioration beginning in mid-life. We have now examined and investigated more than 60 alkaptonuria patients from a clinical, biochemical, and molecular perspective. We have identified 90 percent of the genetic mutations causing alkaptonuria in our patients and have described a man with alkaptonuria and diabetic nephropathy whose renal disease exacerbated his ochronosis. We also found three individuals with darkened ear cartilage as a consequence of prolonged use of the antibiotic minocycline, who carried the diagnosis of alkaptonuria. Two women with alkaptonuria were given low doses of nitisinone, a drug that inhibits the enzyme producing homogentisic acid. Although their plasma tyrosine levels rose dramatically, the lowering of homogentisic acid production provides proof of principle for the use of nitisinone in alkaptonuria.

### Other Metabolic Disorders

*Gahl, Huizing, Anikster, Bernardini, Lalor, Kleta; in collaboration with Skovby*

We also investigate a variety of other rare disorders of human metabolism. Members of the section have identified the gene and the mutations responsible for type III 3-methylglutaconic aciduria in Iraqi-Jewish and non–Iraqi-Jewish patients. A family with gray platelet syndrome has been described and characterized. Members of the section have reported a patient with Griscelli syndrome and neurological involvement caused by mutations in *rab27a* rather than the expected gene, *myosin 5A*. In collaboration with other investigators, we have reported patients with lysosomal free sialic acid storage due to mutations in the gene encoding the lysosomal sialic acid transporter. The section remains the world's premier referral laboratory for diagnosing sialuria and other disorders of free sialic acid metabolism.

## SELECTED PUBLICATIONS

1. Anikster Y, Huizing M, Anderson PD, Fitzpatrick DL, Klar A, Gross-Kieselstein E, Berkun Y, Shazberg G, Gahl WA, Hurvitz H. Evidence that Griscelli syndrome with neurological involvement is caused by mutations in *rab27A*, not *myo5A*. Am J Hum Genet. 2002;71:407-414.
2. Anikster Y, Huizing M, White J, Bale S, Gahl WA, Toro J. Mutation of a new gene causes a unique form of Hermansky-Pudlak syndrome in a genetic isolate of central Puerto Rico. Nat Genet. 2001;28:376-380.
3. Anikster Y, Kleta R, Shaag A, Gahl WA, Elpeleg O. Type III 3-methylglutaconic aciduria (Optic Atrophy Plus Syndrome, or Costeff Optic Atrophy Syndrome): identification of the *OPA3* gene and its founder mutation in Iraqi Jews. Am J Hum Genet. 2001;69:1218-1224.
4. Falik-Zaccai TC, Anikster Y, Rivera CE, Horne MK III, Schliamser L, Phornphutkul C, Attias D, Gahl WA. A new genetic isolate of Gray Platelet Syndrome (GPS): clinical, cellular and hematologic characteristics. Mol Genet Metab. 2001;74:303-313.
5. Gahl WA, Brantly M, Troendle J, Avila NA, Padua A, Montalvo C, Cardona H, Calis KA, Gochuico B. Effect of pirfenidone on the pulmonary fibrosis of Hermansky-Pudlak syndrome. Mol Genet Metab. 2002;76:234-242.
6. Gahl WA, Thoene J, Schneider JA. Cystinosis: a disorder of lysosomal membrane transport. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Vogelstein B, eds. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill. 2001;5085-5108.
7. Gahl WA, Thoene JG, Schneider JA. Cystinosis. N Engl J Med. 2002;347:111-121.
8. Huizing M, Anikster Y, Fitzpatrick DL, Jeong AB, D'Souza M, Rausche M, Kaiser-Kupfer MI, White JG, Gahl WA. Hermansky-Pudlak syndrome type 3 in Ashkenazi Jews and other non-Puerto Rican patients with hypopigmentation and platelet storage pool deficiency. Am J Hum Genet. 2001;69:1022-1032.
9. Huizing M, Gahl WA. Disorders of vesicles of lysosomal lineage: the Hermansky-Pudlak syndromes. Curr Mol Med. 2002;2:451-467.

10. Huizing M, Saranjarajan R, Strovel E, Zhao Y, Gahl WA, Boissy RE. AP-3-dependent vesicles carry tyrosinase, but not TRP-1, in cultured human melanocytes. Mol Biol Cell. 2001;12:2075-2085.
11. Introne WJ, Phornphutkul C, Bernardini I, McLaughlin K, Fitzpatrick D, Gahl WA. Exacerbation of the ochronosis of alkaptonuria due to renal insufficiency. Mol Genet Metab. 2002;77:136.
12. Kleta R, Anikster Y, Lucero C, Shotelersuk, V, Huizing M, Bernardini I, Park M, Thoene J, Schneider J, Gahl WA. *CTNS* mutations in African American cystinosis patients. Mol Genet Metab. 2001;74:332-337.
13. Kleta R, Skovby F, Christensen E, Rosenberg T, Gahl WA, Anikster Y. 3-Methylglutaconic aciduria type III in a non Iraqi-Jewish kindred: clinical and molecular findings. Mol Genet Metab. 2002;76:201-106.
14. Phornphutkul C, Anikster Y, Huizing M, Braun P, Brodie C, Chou JY, Gahl WA. The promoter of a lysosomal membrane transporter gene, *CTNS*, binds Sp-1, shares sequences with the promoter of an adjacent gene (*CARKL*), and causes cystinosis if mutated in a critical region. Am J Hum Genet. 2001;69:712-721.
15. Tsilou E, Csaky K, Rubin BI, Gahl W, Kaiser-Kupfer M. Retinal visualization in an eye with corneal crystals using indocyanine green videoangiography. Am J Ophthalmol. 2002;134:123-125.

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# HERITABLE NEURODEGENERATIVE AND INFLAMMATORY/AUTOIMMUNE DISODERS

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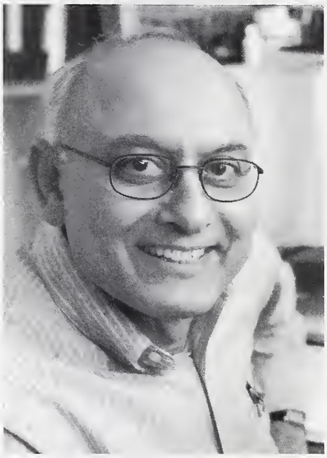
Bhabadeb Chowdhury, Ph.D., *Postdoctoral Fellow*
Asim K. Mandal, Ph.D., *Postdoctoral Fellow*[a]
Aperna Mital, Ph.D., *Postdoctoral Fellow*
Rabindranath Ray, Ph.D., *Postdoctoral Fellow*
Mary A. Anderson, M.A., *Technical Training Fellow*[b]
Pei Chen Tsai, M.S., *Technical Training Fellow*
Sondra Levin, M.D., *Guest Researcher*
Aprile Pilon-Clayton, Ph.D., *Guest Researcher*



We conduct both basic and clinical investigations on the molecular mechanisms of hereditary neurodegenerative and inflammatory/autoimmune diseases in order to develop novel approaches for the treatment of these diseases. Our studies focus on understanding the regulation and physiological functions of several genes and their products, including uteroglobin (UG), soluble phospholipases $A_2$ ($sPLA_{2s}$), palmitoyl-protein thioesterase (PPT), and neutral ceramidase. UG is a multifunctional secreted protein with potent $sPLA_2$-inhibitory and immunomodulatory activities. We demonstrated that UG-deficient mice, which we generated, suffer from IgA-nephropathy, the most common primary renal glomerular disease, for which there is no effective treatment. In a related investigation, we study the PPT gene because inactivating mutations in this gene cause infantile neuronal ceroid lipofuscinosis (INCL), a severe neurodegenerative disease of childhood, for which there is no effective treatment. This hereditary disease belongs to a group of common genetic neurodegenerative disorders called Batten disease. Currently, we are conducting a pilot study to determine whether Cystagon™ may be an effective treatment for INCL. We are also using a mouse model of INCL to understand the molecular mechanisms in further detail and to develop novel therapeutic approaches that use gene transfer and embryonic stem (ES) cell technologies.

### Role of a Novel Innate Homeostatic Mechanism in Counteracting Airway Inflammation and Protecting against Allergic Asthma

*Mandal, Zhang, Chowdhury, Ray, Mukherjee; in collaboration with Pattabiraman*

In allergic asthma, signalling via the prostaglandin $D_2$ receptor DP mediates airway inflammation by unknown mechanisms. Using three different cell types (i.e., fibroblasts and epithelial and smooth muscle cells) that are constituents of the respiratory system, we demonstrated that DP signalling mediates the activation of nuclear factor (NF)-κB via p38 MAPK, p42/44 MAPK, and PKC pathways in a cell-specific manner, stimulating the expression of COX-2, which is essential for the production of pro-inflammatory PGs. We found that $PGD_2$-induced activation of NF-κB and stimulation of COX-2 expression are drastically inhibited by UG, a low molecular weight, anti-inflammatory/anti-chemotactic protein secreted by the tracheobronchial epithelia of virtually all vertebrates, a most important discovery. Molecular modeling studies indicate that a central hydrophobic cavity created by the UG homodimer sequesters $PGD_2$, abrogating DP signalling (Fig. 13A and B). Finally, using a UG-deficient mouse model of allergic asthma, we demonstrated that pretreatment of these mice with purified recombinant UG (rUG) before

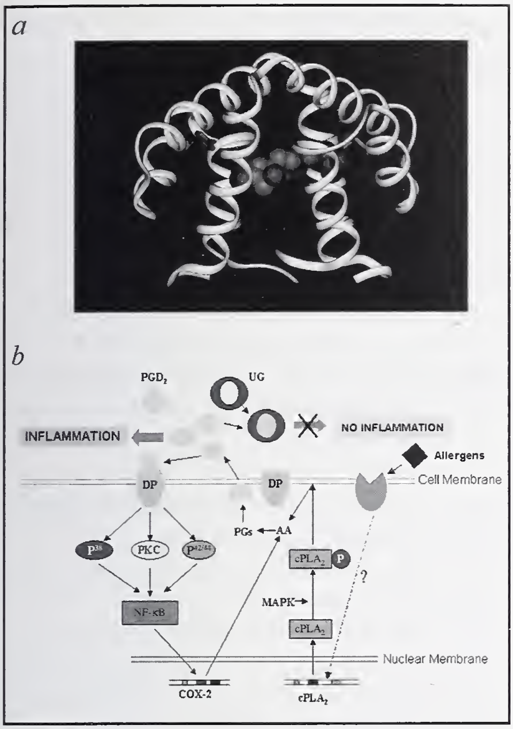


**FIGURE 13**

A MOLECULAR MODEL FOR DP-SIGNALLING AND ITS REGULATION BY UG

**Panel a:** Energy-minimized structure of PGD$_2$ docked into the central cavity of the dimer crystal structure of recombinant human UG. The natural form of UG is a homodimer. The human UG dimer structure is represented as ribbons and PGD$_2$ as a van der Waals (space-filling) model. In this figure only the lowest energy structure of the human UG-PGD$_2$ complex is shown. One of the two symmetrically related tyrosines (Y21) in the UG dimer forms a hydrogen bond with the carboxyl group of PGD$_2$ and with the carbon atoms between the two carbon chains, but not with either the hydroxyl oxygen or with the carbonyl oxygen. **Panel b:** A schematic model of a novel innate mechanism of homeostasis: the molecular mechanism of DP signaling and the effect of UG. We propose that PGD$_2$ binds to the G-protein coupled receptor, DP, and transduces signals in an autocrine as well as paracrine manner. Mediated by p38 MAPK, p42/44MAPK and PKC, PGD$_2$-DP interaction activates NF-κB in a cell-specific manner, which up-regulates the expression of COX-2 that catalyzes the production of proinflammatory prostaglandins from arachidonic acid (AA); cPLA$_2$, whose expression is reported to be stimulated by antigens or allergens and its activation mediated by MAPK, catalyzes the release of AA from cell membrane phospholipids once phosphorylated and activated cPLA$_2$ has been translocated from the cytosol. UG binds and possibly sequesters PGD$_2$, thereby blocking its interaction with DP and preventing the initiation and/or propagation of the inflammatory response.

allergen challenge prevents COX-2 expression and eosinophil infiltration in the lungs, confirming the results of our *in vitro* experiments. We propose that DP signalling mediates airway inflammation by NF-κB activation, leading to the expression of one of its target genes, COX-2, which is critical for the production of pro-inflammatory prostaglandins. In this scenario, UG is an essential component of a novel, innate homeostatic mechanism that counteracts inadvertent activation of an allergen-induced inflammatory response in this vital organ and prevents allergic asthma.

### Altered Pulmonary Eosinophilic Inflammation in Uteroglobin-Deficient Mice

*Zhang, Mukherjee; in collaboration with Huang*

The role of UG in modulating pulmonary allergic inflammation was examined in UG-deficient mice generated by targeted gene disruption. Wild-type and homozygous (UG$^{-/-}$) mice were sensitized with ovalbumin (OVA) and challenged with either OVA or saline. When compared with wild-type mice, OVA-sensitized and challenged UG-/- mice exhibited a significantly higher level of pulmonary eosinophilia as well as significantly increased plasma levels of Th2 cytokines (i.e., IL-4, IL-5, IL-9, and IL-13). In addition, UG-deficient mice exhibited an increased level of eotaxin, but not RANTES, whereas we detected no significant difference in the level of the Th1 cytokine IFN. The results provided the first *in vivo* evidence that UG plays a role in the modulation of pulmonary allergic inflammation.

### Molecular Mechanism of Inhibition of Phospholipase A$_2$ Activity by Uteroglobin

*Chowdhury, Zhang, Mukherjee*

UG is an anti-inflammatory, secreted protein that inhibits soluble phospholipase A$_2$ (sPLA$_2$) by an unknown mechanism. It is a homodimer, in which each of the 70-amino acid subunits forms four α-helices. We previously reported that the sPLA$_2$-inhibitory activity of UG might reside in a segment of α-helix 3 that is exposed to the solvent. In addition, it has been suggested that UG may inhibit sPLA$_2$ activity by binding and sequestering Ca$^{2+}$, which is essential for sPLA$_2$ activation. By site-specific mutation, we demonstrated that Lys 43 Glu, Asp 46 Lys, or a combination of the two mutations in the full-length, recombinant human UG (rhUG) abrogates its $sPLA_2$-inhibitory activity. We demonstrated further that recombinant UG does not bind to $Ca^{2+}$, although when it is expressed with histidine-tag (H-tag), it is capable of binding to $Ca^{2+}$. Taken together, our results show that Lys 43 and Asp 46 in rhUG are critical residues for the $sPLA_2$-inhibitory activity of UG and that $Ca^{2+}$-sequestration by rhUG is not likely to be one of the mechanisms responsible for its $sPLA_2$-inhibitory activity.

### Clinical Trial of Recombinant Uteroglobin for the Treatment of Neonatal Respiratory Distress Syndrome

*Pilon-Clayton, Chowdhury, Zhang, Choi, Mukherjee*

Neonatal Respiratory Distress Syndrome (NRDS) is a surfactant deficiency disease that develops in a significant number of premature neonates. Although surfactant replacement therapy is highly successful in resuscitating these neonates, some patients go on to develop a chronic lung disease known as bronchopulmonary dysplasia (BPD). One potential mechanism of pathogenesis of BPD is degradation of phospholipids in the surfactant, causing the production of pro-inflammatory lipid mediators (e.g., prostaglandins and leukotrienes). Given that UG is naturally present in the newborn and adult lungs but absent from those of premature neonates and is an anti-inflammatory protein, we sought to determine whether intratracheal administration of recombinant human UG (rhUG) is efficacious in treating NRDS and preventing BPD.

The most important milestone in 2002 was, therefore, the completion of this Phase I clinical study of recombinant human uteroglobin (also known as rhCC10) for prevention of bronchopulmonary dysplasia in premature infants with respiratory distress syndrome. In this study, conducted under a collaborative research and development agreement (CRADA) by Claragen Inc., 22 patients were enrolled in three groups: those given a placebo, those given 1.5 mg/kg rhUG, and those given 5 mg/kg rhUG. Despite a higher incidence of necrotizing enterocolitis in the treated groups, the condition was not attributable to the study drug. No issues with safety or tolerability of the rhCC10 were observed. Considerable progress has been made on the GMP manufacturing process for the rhCC10 drug product, increasing yield six-fold by changing the bacterial expression strain as well as by altering the purification procedure to reduce bacterial contaminants and CC10 aggregates. Clinical development of rhCC10 for prevention of neonatal BPD with a Phase II trial as well as further preclinical work for the application of rhCC10 in adult respiratory distress syndrom and asthma will be carried out in the coming year.

### Benefit of Cystagon™ for Infant Neuronal Ceroid Lipofuscinoses Patients

*Levin, Zhang, Caruso, Gropman, Mukherjee; in collaboration with Fischbeck*

Neuronal ceroid lipofuscinoses (NCLs) are the most common (1 in 12,500) heritable progressive encephalopathies of children. Infantile NCL (INCL) is caused by lysosomal PPT deficiency. PPT catalyzes the hydrolysis of thioester linkages in S-acylated polypeptides, and a deficiency causes abnormal accumulation of the polypeptides, leading to INCL. Given that thioester bonds are susceptible to nucleophilic attack, drugs with nucleophilic properties (e.g., Cystagon™ may have therapeutic potential for INCL. Last year, we demonstrated that Cystagon™ not only disrupts thioester linkages in S-acylated polypeptides in cultured cells from INCL patients but also mediates the depletion of intracellular ceroid deposits and prevents their reaccumulation. Taken together, the results suggest that the drug could be an effective treatment for INCL. Given that INCL is a uniformly fatal disease, for which currently there is no effective treatment, and that the active compound of phosphocysteamine has been in clinical use for more than two decades with a proven record of safety, we have implemented a pilot study to determine whether cysteamine bitartrate (Cystagon™) is beneficial for INCL patients. We have received regulatory approval for treating five INCL patients in this study. So far, we have recruited two patients to the protocol, and preliminary evaluations indicate stabilization of retinal functions and of cortical degeneration. The most dramatic results were obtained from the electron microscopic analyses of the white blood cells of these patients. Compared with the cells before treatment, the mononuclear cells show virtually no ceroid depositions in their lysosomes after the initiation of Cystagon™ treatment. So far, the patients do not require any antiepileptic medications. No adverse effects have been encountered with the treatment.

### Maintenance of Integrity of Gastrointestinal and Renal Cells by Neutral Ceramidase, Which Prevents Ceramide-Induced Apoptosis

*Choi, Anderson, Zhang, Ray, Mukherjee; in collaboration with Popescu*

Ceramide, a natural dietary constituent of our daily diet, is a mediator of apoptosis in many cell types. This sphingolipid may also disrupt the mucosal integrity of the gastrointestinal tract by stimulating apoptosis. However, the mechanism that prevents ceramide-induced apoptosis is poorly understood, although it may involve a nonlysosomal ceramidase in the gastrointestinal tract. The enzyme manifests optimal activity in the neutral to alkaline pH (neutral/alkaline ceramidase), degrading ceramide into sphingosine and free fatty acid. In this study, we characterized the murine neutral ceramidase gene, mapped its chromosomal location, determined the tissue-specific expression, and delineated its role in ceramide-induced apoptosis using both *in vitro* and *in vivo* systems. Our results show that the murine neutral ceramidase (N-CDase) gene structure is virtually identical to that of the human N-CDase. It is expressed at high levels in mouse kidneys and in specific segments of the upper gastrointestinal tract. We also found that neutral ceramidase mRNA expression is stimulated by its substrate, C2-ceramide. Most important, we found that feeding mice with C2-ceramide or injecting them with a specific inhibitor of neutral ceramidase before feeding ceramide dramatically increased apoptosis in mesangial cells *in vitro* and in murine gastrointestinal tract *in vivo*. We propose that neutral ceramidase is part of a novel homeostatic mechanism for preventing apoptosis in vital organs such as the gastrointestinal tract and the kidneys.

## SELECTED PUBLICATIONS

1. Chen LC, Zhang Z, Myers AC, Huang SK. Cutting edge: altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-kDa protein. J Immunol. 2001;167:3025-3028.
2. Chowdhury B, Mantile-Selvaggi G, Miele L, Cordella-Miele E, Zhang Z, Mukherjee AB. Lys 43 and Asp 46 in alpha-helix 3 of uteroglobin are essential for its phospholipase $A_2$ inhibitory activity. Biochem Biophys Res Commun. 2002;295:877-883.
3. Mandal AK, Zhang Z, Chou J, Mukherjee AB. Pancreatic phospholipase $A_2$ via its receptor regulates the expression of key enzymes of phospholipid and sphingolipid metabolism. FASEB J. 2001;15:1834-1836.
4. Wang CY, Lei HJ, Huang CY, Zhang Z, Mukherjee AB, Yuan CJ. Induction of cyclooxygenase-2 by staurosporine through the activation of nuclear factor for IL-6 (NF-IL6) and activator protein 2 (AP2) in an osteoblast-like cell line. Biochem Pharmacol. 2002;64:177-184.
5. Zhang Z, Butler JD, Levin SW, Wisniswski K, Brooks SS, Mukherjee AB. Lysosomal ceroid depletion by drugs: therapeutic implications for a hereditary neurodegenerative disease of childhood. Nat Med. 2001;7:478-484.

[a] Left NICHD in 2001.

[b] Left NICHD in 2002.

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# MOLECULAR BIOLOGY OF DYSMORPHOLOGY

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We study the molecular, biochemical, and cellular processes that underlie dysmorphic syndromes and birth defects. Our work currently focuses on the inborn errors of cholesterol synthesis, including Smith-Lemli-Opitz syndrome SLOS, an autosomal recessive, multiple malformation syndrome characterized by dysmorphic facial features, mental retardation, hypotonia, poor growth, and variable structural anomalies of the heart, lungs, brain, limbs, gastrointestinal tract, and genitalia. The SLOS phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die due to multiple major malformations. In contrast, at the mild end of the phenotypic spectrum, SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol to cholesterol.

### Smith-Lemli-Opitz Syndrome

*Correa-Cerro, Cozma, Krakowiak, Wassif, Wright, Porter*

Our laboratory initially cloned the human 3β-hydroxysterol $\Delta^7$-reductase gene (*DHCR7*) and demonstrated mutations of the gene in SLOS patients. To date, over 70 different mutations of *DHCR7* have been identified in SLOS patients. In support of our clinical protocol "Clinical and Basic Investigations into Smith-Lemli-Opitz Syndrome," we have genotyped over 50 SLOS patients and have continued to identify novel mutations of the gene. To further our understanding of the mechanisms underlying the broad phenotypic spectrum in this human malformation syndrome, we have used deuterium oxide labeling to measure residual DHCR7 activity in fibroblasts from patients with known genotypes and well-characterized phenotypes. The most common SLOS mutation, IVS8-1G→C, is a single nucleotide G-to-C change at the -1 position of the splice acceptor in the eighth intron. Aberrant splicing to a cryptic splice acceptor results in the insertion of 134 base pairs of intronic sequence into the mRNA. This mutation results in an allele with no enzymatic function and accounts for about one-third of the identified mutant alleles. The second most common SLOS mutation is T93M. Other common mutations include W151X, V326L, R404C, and R352W. SLOS may be more common than typically thought. The carrier frequency for the IVS8-1G→C allele is on the order of 1 percent in Caucasians. Surprisingly, although few patients of African heritage have been identified, the carrier frequency for the same allele in African Americans was found to be 0.73 percent. These carrier frequencies predict a disease incidence for SLOS of at least 1/40,000 and 1/75,000 in Caucasians and African Americans, respectively.

### Murine Models for SLOS

*Correa-Cerro, Wassif, Porter*

Using gene targeting in murine embryonic stem cells, we have produced three SLOS mouse models, including a null mutation and two hypomorphic point mutations. The point mutations, which have been described in human patients, are T93M and L99P. Mouse pups that are homozygous for the null mutation, similar to human patients, have variable craniofacial anomalies, are growth-retarded, feed poorly, and appear weak. The pups die during the first day of life due to failure to feed. Biochemical characterization shows that the pups have markedly elevated serum and tissue 7-dehydrocholesterol (7-DHC) levels as well as reduced serum and tissue cholesterol levels. Cleft palate was present in 9 percent of the *Dhcr7-/-* pups and is found in approximately one-third of all SLOS patients. To characterize further the neurological abnormalities seen in the mutant mouse pups, we measured the response of cortical neurons to the neurotransmitters GABA and glutamate. Comparing mutant with control neurons, we observed no significant difference in the response to GABA, whereas the glutamate response of mutant neurons decreased significantly. A decreased glutamate response is consistent with the phenotypic observation of poor feeding by the mutant animals. Glutamate receptors are involved in neuronal pattern formation, long-term potentiation and depression, memory acquisition, and learning; neurological dysfunctions, including poor feeding, hypotonia, mental retardation, and behavioral problems, are major clinical problems in SLOS. The impaired glutamate response observed in the mouse model may yield insight into the etiology of some of the neurological dysfunction seen in SLOS. One limitation of the null SLOS mouse model is that it dies during the first day of life, rendering it unsuitable for the study of therapeutic interventions for SLOS. We produced two hypomorphic mouse mutations, T93M and L99P, to avoid this limitation. The mice are currently being characterized for phenotypic, biochemical, neuromuscular, neurological, and behavioral abnormalities.

### Clinical Studies in SLOS Patients

*Porter*

In addition to basic research designed to understand the pathophysiological processes underling SLOS, we have initiated a clinical protocol to study genotype/phenotype correlations, endocrinological aspects, and neurological, dental, speech, and behavioral aspects of SLOS. To date, we have enrolled over 40 SLOS patients. Therapy for SLOS includes dietary cholesterol supplementation. Even though we have noted improvements in growth and behavior, the long-term effect, if any, of dietary cholesterol supplementation on the nervous system remains to be seen. We are investigating whether an MRI enhancement technique known as Magnetic Transfer Imaging (MTI), which is highly sensitive to changes in myelin, can provide a quantitative measure of abnormal myelin in SLOS patients and thus be used to monitor therapeutic interventions. We found elevated MTI values in SLOS patients compared with controls. In addition, in contrast to normal pediatric patients in whom maximum MTI values are observed by 20 months of age, MTI values show an age-dependent increase in SLOS patients up to 10 years of age. We are continuing to obtain longitudinal serial data to determine if MTI will provide a tool for monitoring future therapeutic interventions. Endocrine investigations have shown that about half our SLOS patients have compensated adrenal insufficiency, a finding that will affect their clinical management. We have also made progress in another area by defining a specific behavioral phenotype associated with SLOS that includes autistic and obsessive-compulsive traits.

### Lathosterolosis

*Cozma, Krakowiak, Wassif, Porter*

Lathosterol 5-desaturase catalyzes the conversion of lathosterol to 7-dehydrocholesterol, the enzymatic step immediately preceding the defect in SLOS. Thus, in order to further our understanding of the relative roles of decreased cholesterol and increased 7-dehydrocholesterol in SLOS, we disrupted the mouse lathosterol 5-desaturase gene (*Sc5d*) by using targeted homologous recombination in embryonic stem cells. The *Sc5d-/-* pups are stillborn and have micrognathia, cleft palates, and limb patterning defects. Many of the malformations in the mutant mice resemble malformations found in SLOS and are consistent with impaired *hedgehog* signalling during development. Biochemically, the mice have markedly elevated serum and tissue lathosterol levels and decreased cholesterol levels.

One goal of producing a lathosterolosis mouse model was to gain phenotypic insight to help identify a corresponding human malformation syndrome. We have now identified a human patient (an infant) with lathosterolosis. The human malformation syndrome has not been described previously. Biochemically, fibroblasts from the patient show decreased cholesterol and increased lathosterol levels. Mutation analysis showed that the patient is homozygous for a single A-to-C nucleotide change at position 137 in *SC5D*, resulting in a mutant enzyme in which the amino acid serine is substituted for tyrosine at position 46. Both parents were heterozygous for the mutation. Phenotypically, the infant resembled severe SLOS. Malformations found in both the human patient and the mouse model include growth failure, abnormal nasal structure, abnormal palate, micrognathia, and postaxial polydactyly. One unique aspect of lathosterolosis is the clinical finding of mucolipidosis in the affected infant. This clinical presentation is not reported in SLOS and may help in clinically separating the two disorders. This lysosomal storage disorder can be replicated in embryonic fibroblasts from the *Sc5d* mutant mouse model.

### Characterization of LIM Homeobox Genes *Lhx2* and *Lhx9*

*Wassif, Porter*

*Lhx2* and *Lhx9* are two closely related LIM homeobox genes that are essential for the development of multiple organ systems. *Lhx2* mutant mice are anophthalmic, exhibit forebrain malformations, and die *in utero* due to inefficient definitive erythropoiesis. Recent work has shown that Lhx2 functions to pattern the dorsal telencephalon. *Lhx9* has an overlapping but a distinct expression pattern compared with *Lhx2*. Lhx9 mutant mice are agonadal, leading to the conclusion that Lhx9 is essential for gonad development. In collaboration with LMGD, NICHD, GDRB, and NHGRI, we are analyzing *Lhx2/Lhx9* compound mutants to determine the combined functions of the two genes. Our group's characterization of double *Lhx2* and *Lhx9* mutant embryos has demonstrated that the two LIM homeobox genes are functionally redundant with respect to limb development.

## SELECTED PUBLICATIONS

1. Nowaczyk MJM, Siu VM, Krakowiak PA, Porter FD. Adrenal insufficiency and hypertension in a newborn infant with Smith-Lemli-Opitz Syndrome. Am J Med Genet. 2001;15:223-225.
2. Porter FD. Malformation syndromes due to inborn errors of cholesterol synthesis. J Clin Invest. 2002;110:715-724.
3. Quezado ZMN, Veihmeyer J, Schwartz L, Nwokoro N, Porter FD. Anesthesia and airway management of the pediatric patient with Smith-Lemli-Opitz syndrome. Anesthesiology. 2002;97:1015-1019.
4. Shackleton C, Roitman E, Guo L, Wilson WK, Porter FD. Identification of 7(8) and 8(9) unsaturated adrenal steroid metabolites produced by patients with 7–dehydrosterol-7-reductase deficiency (Smith-Lemli-Opitz syndrome). J Steroid Biochem Mol Biol. 2002;1747:1-8.
5. Wassif CA, Vied D, Tsokos M, Connor WE, Steiner RD, Porter FD. Intracellular LDL cholesterol metabolism is impaired in Smith-Lemli-Opitz syndrome fibroblasts. Mol Genet Metab. 2002;75:325-334.

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Our goal is to understand the molecular genetics and pathogenesis of heritable metabolic human disorders with specific emphasis on type 1 glycogen storage diseases (GSD-I). Also known as von Gierke disease, GSD-I is a group of autosomal recessive disorders caused by deficiencies in the endoplasmic reticulum (ER)–bound glucose-6-phosphatase (G6Pase) system, a key enzyme complex in glucose homeostasis. The G6Pase system comprises at least two integral membrane proteins, G6Pase and the glucose-6-phosphate transporter (G6PT). G6PT translocates glucose-6-phosphate (G6P), the product of gluconeogenesis and glycogenolysis, from the cytoplasm to the lumen of the ER, and, inside the ER, G6Pase catalyzes the hydrolysis of G6P to produce glucose and phosphate. Over the last 20 years, dietary therapy has been used to alleviate some, but not all, metabolic abnormalities of GSD-1 patients and to slow disease progress. However, the underlying disease remains untreated, and poor compliance frequently limits the efficacy of dietary treatment. Therefore, long-term complications still develop in adult patients. An understanding of the molecular genetics and pathogenesis of GSD-1 is needed in order to develop therapies that can rectify the long-term complications of GSD-1.

### Gene Therapy for Glycogen Storage Disease Type Ia

*Sun, Pan, Shieh, Ghosh, Chen, Mansfield, Chou*

Deficiencies in G6Pase and G6PT cause GSD-1a and GSD-1b, respectively. Patients suffering from both disorders manifest the symptoms of G6Pase enzyme deficiency characterized by growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia. However, GSD-Ib patients also present with unique symptoms not so obviously associated with G6Pase activity, including chronic neutropenia and myeloid dysfunctions, which result in recurrent bacterial infections. The most prevalent from of GSD-Ia is caused by a deficiency in G6Pase, a highly hydrophobic protein anchored to the ER by 9-transmembrane helices. The protein cannot be expressed in a soluble form and must embed correctly in the ER membrane and couple with other proteins to be functional. Therefore, enzyme replacement therapy is not an option, but somatic gene therapy, i.e., targeting G6Pase to the liver and the kidney, is an attractive possibility. To this end, we had previously generated G6Pase-deficient mice that manifest a metabolic profile and phenotype virtually identical to that of human GSD-Ia patients. Using neonatal GSD-Ia mice, we now demonstrate that gene transfer mediated by a combined adeno- and adeno-associated virus vector leads to sustained G6Pase expression in both the liver and kidney and corrects the murine GSD-Ia disease for at least 12 months. Our results suggest that human GSD-Ia would be treatable by gene therapy.

### The Catalytic Center of Glucose-6-Phosphatase

*Ghosh, Shieh, Pan, Sun, Chou*

The amino acids constituting the catalytic center of G6Pase include Lys$^{76}$, Arg$^{83}$, His$^{119}$, Arg$^{170}$, and His$^{176}$. During catalysis, a His residue in G6Pase becomes phosphorylated, generating an enzyme-phosphate intermediate. It was predicted that His$^{176}$ would be the amino acid that acts as a nucleophile forming a phosphohistidine-enzyme intermediate and that His$^{119}$ would be the amino acid that provides the proton needed to liberate the glucose moiety. However, the phosphate acceptor in G6Pase has eluded molecular characterization. To identify the His residue that covalently binds the phosphate moiety, we generated recombinant adenoviruses carrying wild-type G6Pase and active site mutants of this enzyme. A 40-kDa [$^{32}$P]-phosphate-G6Pase intermediate was identified after incubating [$^{32}$P]-glucose-6-phosphate with microsomes expressing wild-type enzyme but not with microsomes expressing either H119A or H176A mutant G6Pase. Human G6Pase contains five methionine residues at positions 1, 5, 121, 130, and 279. After cyanogen bromide cleavage, His$^{119}$ is predicted to be within a 116 amino acid peptide of 13.5 kDa with an isoelectric point of 5.3 (residues 6–121) and His$^{176}$ within a 149 amino acid peptide of 16.8 kDa with an isoelectric point of 9.3 (residues 131–279). We show that after digestion of a nonglycosylated [$^{32}$P]-phosphate-G6Pase intermediate by cyanogen bromide, the [$^{32}$P]-phosphate remains bound to a peptide of 17 kDa with an isoelectric point above 9, demonstrating that His$^{176}$ is the phosphate acceptor in G6Pase.

### Structure and Function Analysis of Mutations in Glucose-6-Phosphatase

*Shieh, Pan, Chen, Chou*

To date, 75 *G6Pase* mutations have been identified in GSD-Ia patients: 48 missense, nine nonsense, 15 insertion/deletion, and three splicing mutations. Interestingly, 64 percent of candidate mutations are missense mutations that result in single amino acid substitutions. Characterization of these mutations will provide valuable information on functionally important residues of the protein. Using site-directed mutagenesis and transient expression assays, we have characterized all 48 missense mutations. The database of residual activity retained by these mutants will serve as a reference in evaluating genotype-phenotype relationships and identifying the minimal G6Pase activity required to correct the GSD-Ia phenotype.

### Structure-Function Analysis of the Glucose-6-Phosphate Transporter

*Chen, Pan, Shieh, Chou*

G6PT is anchored in the ER by 10-transmembrane helices. To date, 69 *G6PT* mutations, including 28 missenses and two codon-deletions, have been identified in GSD-Ib patients. We previously characterized one codon deletion and 15 missense mutations by using a pSVL-based expression assay. We now report the functional characterization of all 30 codon mutations by using an improved G6PT assay based on an adenoviral vector–mediated expression system. Twenty of the naturally occurring mutations completely abolish microsomal G6P uptake activity while the other 10 mutations partially inactivate the transporter. We also report a structure-function analysis of G6PT and show that wild-type and mutant G6PT are degraded in cells predominantly through the proteasome pathway.

## SELECTED PUBLICATIONS

1. Chou JY, Matern D, Mansfield BC, Chen YT. Type I glycogen storage diseases: disorders of the glucose-6-phosphatase complex. Curr Mol Med. 2002;2:121-143.

2. Ghosh A, Shieh J-J, Pan C-J, Sun M-S, Chou JY. The catalytic center of glucose-phosphatase: His$^{176}$ is the nucleophile forming the phosphohistidine-enzyme intermediate during catalysis. J Biol Chem. 2002;277:32837-32842.

3. Hiraiwa H, Pan C-J, Lin B, Akiyama TE, Gonzalez FJ, Chou JY. A molecular link between the common phenotypes of type 1 glycogen storage disease and HNF1a-null mice. J Biol Chem. 2001;276:7963-7967.

4. Shieh J-J, Terzioğlu M, Hiraiwa H, Marsh J, Pan CJ, Chen LY, Chou JY. The molecular basis of glycogen storage disease type 1a: structure and function analysis of mutations in glucose-6-phosphatase. J Biol Chem. 2001;277:5047-5053.
5. Sun M-S, Pan C-J, Shieh JJ, Ghosh A, Chen LY, Mansfield BC, Ward JM, Byrne BJ, Chou JY. Sustained hepatic and renal glucose-6-phosphatase expression corrects glycogen storage disease type Ia in mice. Hum Mol Genet. 2002;11:2155-2164.

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# MEMBRANE REMODELING DURING PATHOGENESIS AND THE MECHANISM OF EXOCYTOSIS

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Membrane fusion is a critical for membrane assembly, mitosis, Golgi trafficking, secretion, enveloped viral infection, insulin release, histamine release, and fertilization while membrane fission allows for endocytosis and parasite entry. We attempt to understand the functional pathway of membrane events at the level of physical forces in order to assign roles to putative proteins, mutants, and second messengers. We have detected an intermediate providing ionic continuity between the two aqueous spaces separated before membrane fusion, the fusion pore, which we have continued to study with electrophysiological, molecular, and cellular techniques. We have extended the new methods developed in the study of the fusion pore to the measurement of a fission pore as the object through which parasites, in particular *P. falciparum*, infect cells. We have also discovered a profound and potent destabilization of lipid membranes by protein molecules that promote apoptosis. In addition, we focus on the mechanisms of exocytosis, specifically the cortical fusion of secretory vesicles of the sea urchin egg. By combining physiological and molecular analyses with a variety of purified membrane isolates containing secretory vesicles that fuse to the plasma membrane or to each other, we are characterizing the final steps of calcium-triggered exocytosis.

## Molecules Mediating Apoptosis Make Lipidic Pores

*Basanez, Chanturiya, Galanis; in collaborating with Sharpe, Hardwick*

During apoptotic cell death, cells usually release apoptogenic proteins such as cytochrome c from the mitochondrial intermembrane space. During apoptosis, Bax-type proteins cause permeabilization of the outer mitochondrial membrane to release mitochondrial apoptogenic factors into the cytosol via a poorly understood mechanism. We have proposed that Bax and Delta-N76Bcl-xL (the Bax-like cleavage fragment of Bcl-xL) function by forming pores that are at least partially composed of lipids (lipidic pore formation). Given that the membrane monolayer must bend during lipidic pore formation, we explored the effect of intrinsic membrane monolayer curvature on pore formation. Nonlamellar lipids with positive intrinsic curvature such as lysophospholipids promoted membrane permeabilization, whereas nonlamellar lipids with negative intrinsic curvature such as diacylglycerol and phosphatidylethanolamine inhibited membrane permeabilization. The differential effects of nonlamellar lipids on membrane permeabilization did not correlate with lipid-induced changes in membrane binding or insertion of Bax or Delta-N76Bcl-xL. Taken together, these results are consistent with (but do not prove) the notion that Bax-type proteins change the bending propensity of the membrane to form pores consisting at least in part of lipids in a structure of net positive monolayer curvature.

### New Method for Culturing *Plasmodium falciparum*, the Causative Agent of Malaria

*Li, Glushakova, Chanturiya, Yin*

While the traditional method of culturing *Plasmodium falciparum* has been highly successful, plasmodia replicate poorly in erythrocyte densities with a hematocrit greater than 20 percent. We developed a new method of culturing the major malaria parasite by using a hollow fiber bioreactor that preserves healthy erythrocytes at a hematocrit of up to 100 percent. *Plasmodium falciparum* replicated equally well at all densities studied. The method proved advantageous for large-scale preparation of parasitized erythrocytes (and potentially immunogens thereof) in that high yields (about $10^{10}$ in four days, about $10^{11}$ in a week) could be prepared with less cost and labor than conventional methods. Concomitantly, secreted proteins are concentrated by molecular sieving during culture, perhaps contributing to the parasitemic limit of 8 to 12 percent with 3D7 strain. Our finding that *Plasmodium falciparum* can replicate at packed densities suggests that the method may be useful for studying the pathogenesis of the fatal disease cerebral malaria, which features densely packed blood cells in brain microvasculature.

### Mechanism of Influenza Virus Entry during Infection

*Frolov, Kumar, Verma, Biswas, Alfers, Babcock, Bradow, Farrington, Yin; in collaboration with Reese*

All enveloped viruses inject their genome into the cytoplasm by fusing their envelope membrane with that of the host. In cases such as influenza virus infection, the fusion follows endocytic uptake of a virus particle into a cellular vesicle, which then transports the viral particle into the cell. The size, shapes, and membrane composition of the vesicle affect viral fusion efficiency. Our experimental strategy is to approach vesicle formation and transport and then fusion as sequential stages of a single process. We developed high-resolution admittance techniques in combination with low light–level fluorescent microscopy to detect the formation of individual cellular vesicles and the pinching off of such vesicles into the cellular interior. We resolved the evolution, from initial budding to pinching off, of single vesicles capable of carrying individual influenza virus particles. Determination of the lipid composition of such vesicles and the detection of the transport of actual virus particles in the vesicles are in progress. By developing a system to monitor the fusion of a single virus particle with a model membrane (with a controlled composition), we were able to resolve individual fusion events following viral particle uptake. Our experiments revealed that acidification of the virus interior (normally going through the M2 channel in the viral membrane) is crucial for the fusion pore expansion and thus for the RNA release and ultimate infection. The antivirus drugs amantadine and remantadine, known inhibitors of the M2 channel, do not affect fusion pore formation by the viral fusion protein hemagglutinin, but they do prevent fusion pore expansion. Thus, a further pH-sensitive viral protein besides the classical fusion protein hemagglutinin is involved in the completion of fusion.

### Kinetics and Calcium Dynamics of Exocytosis

*Blank, Hess; in collaboration with Rahamimoff*

We are investigating the mechanisms of calcium-triggered exocytosis, the ubiquitous eukaryotic process by which vesicles fuse to the plasma membrane and release their contents. Although the relationship between exocytosis and calcium is fundamental both to synaptic and nonneuronal secretory function, analysis is problematic because of the temporal and spatial properties of calcium and the fact that vesicle transport, priming, retrieval, and recycling are coupled. By analyzing the kinetics of sea urchin egg secretory vesicle exocytosis *in vitro,* we can resolve the final steps of exocytosis. These steps are modeled as a three-state system: activated, committed, and fused, in which interstate transitions are determined by the probabilities that an active fusion complex commits (alpha) and that a committed fusion complex results in fusion, *p*. The number of committed complexes per vesicle docking site is a Poisson distribution with mean n-bar. Experimentally, *p* and n-bar increase with increasing calcium concentration, whereas alpha and the p/n-bar ratio remain constant, reducing the kinetic description to only one calcium-dependent, controlling variable, n-bar. On average, the calcium dependence of n-bar describes the calcium dependence of the maximum rate (Rmax) and the time to reach Rmax (Tpeak). Thus, the nonlinear relationship between the free calcium concentration and the rate of exocytosis can be explained solely by the calcium dependence of the distribution of fusion complexes at vesicle docking sites.

**Biochemistry and Components of Exocytosis**
*Bezrukov, Backlund, Blank, Humphrey, Verma, Whalley; in collaboration with Yergey, Coorssen*
Although Western blotting is widely used for the detection of specific proteins, it is often thought to be an inadequate technique for accurate measurements of protein concentration. In fact, the analysis of calcium-triggered exocytosis requires an unambiguous identification and quantitative assessment of the membrane surface density of specific molecules. Newly refined immunoblotting and analysis approaches permit a quantitative analysis of the SNARE protein complement (VAMP, SNAP-25, and syntaxin) of functional secretory vesicles. The method illustrates the feasibility of the routine quantification of femtomole to attomole amounts of known proteins by immunoblotting. The results indicate that sea urchin egg secretory vesicles and synaptic vesicles have a marked similarity in SNARE densities.

**Membrane Fusion and Fission: Theoretical Considerations**
*Zimmerberg, Frolov; in collaboration with Chizmadzhev, Kuzmin, Huttner*
While continuing our theoretical work on membrane fusion during exocytosis, we extended it to the processes of membrane fission, which is essential to endocytosis. We study the ways that physical forces and cell membrane inhomogeneities are coordinated to allow controlled and organized vesiculation in the general vacuolar system of cells, the release of infectious viral particles, and the internalization of membrane-bound material. The biochemical and biophysical mechanisms of membrane remodeling are dependent on the composition of a small piece of membrane that rolls up into a new biological entity. Consideration of the structure of these lipid microdomains is important to understanding the mechanisms of membrane budding and fission.

Many investigators believe that microdomains of ordered lipids exist in both leaflets of a lipid bilayer, explaining the effects of lipid composition on cytoplasmic leaflet signalling. Moreover, the coordination of these two leaflets into a "bilayer raft" is an attractive, albeit unproven, idea. If one accepts that the inner and outer leaflets of a microdomain can independently assemble or disassemble and that these more ordered structures have less hydrocarbon chain dispersity, then microdomains should be higher in density than non-raft areas. Thus, the area occupied by each phospholipid head group will decrease in such areas. If one leaflet exists as a raft while the other does not, then the difference in areas would cause the membrane to curve as long as there is no flip-flop of membrane compartments to relieve the asymmetry of area and as long as lipid diffusion is restricted by molecular fences. Thus, control of monolayer order could regulate membrane bending, leading to the provocative suggestion that transmembrane signalling may proceed through monolayer ordering. In a non-ordered membrane, ordering one leaflet may lead to the ordering of the *trans* leaflet through the same forces that would stabilize bilayer rafts. The *cis* leaflet order conveys information, and it can cause protein aggregation onto the newly ordered *trans* leaflet. For example, polymerization of specialized protein domains that bind specifically to the phosphoinositol biphosphate (PIP2) of the inner leaflet raft lipid can lead to PIP2 aggregation, which may order the internal leaflet and in turn order the outer leaflet, perhaps then aggregating extracellular (or lumenal) domains. In this way, proteins on clathrin that bind to PIP2 may cause cargo assembly into a nascent bud. It is thus possible to build a mechanism for trans-bilayer signalling that need not involve protein transmembrane domains.

If microdomains are sitting on either side of a neck, they are effectively in a ring topology; that is, if a vesicle buds out of a membrane from a large domain (a 150-nm-diameter raft has more than twice the area of a 50-nm vesicle), then the donor membrane is left with the hitherto unconsidered geometry of a ring. In other words, dynamin, which resides outside the bilayer, would have a counterpart in the bilayer, a "ring raft." The ring could facilitate fission, as discussed above. In addition, given that rafts must adapt their three-dimensional geometry to the needs of the dynamic situation and change, for example, from tubes to cups to spheres and so forth, then it is important to consider the differential effects of curvature and geometry on ordered and disordered membrane domains. Ring rafts can also play an important role as barriers to lipid diffusion, thus facilitating fission by reducing the number of lipid molecules involved in the fission reaction. Ultimately, lipid microdomain topology must be regulated and organized by the underlying cellular architecture and organizing principles.

## SELECTED PUBLICATIONS

1. Basanez G, Zhang J, Chau BN, Maksaev GI, Frolov VA, Brandt TA, Burch J, Hardwick JM, Zimmerberg J. Pro-apoptotic cleavage products of Bcl-xL form cytochrome c-conducting pores in pure lipid membranes. J Biol Chem. 2001;276:31083-31091.
2. Basanez G, Zimmerberg J. HIV and apoptosis death and the mitochondrion. J Exp Med. 2001;193:F11-14.
3. Blank PS, Vogel SS, Malley JD, Zimmerberg J. A kinetic analysis of calcium-triggered exocytosis. J Gen Physiol. 2001;118:145-156.
4. Chizmadzhev YA, Kuzmin PI, Kumenko DA, Zimmerberg J, Cohen FS. Dynamics of fusion pores connecting membranes of different tensions. Biophys J. 2000;78:2241-2256.
5. Coorssen JR, Blank PS, Albertorio F, Bezrukov L, Kolosova I, Backlund PS, Zimmerberg J. Quantitative femto- to attomole immunodetection of regulated secretory vesicle proteins critical to exocytosis. Anal Biochem. 2002;307:54-62.
6. Desai SA, Bezrukov SM, Zimmerberg J. A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. Nature. 2000;406:1001-1005.
7. Frolov VA, Cho MS, Bronk P, Reese TS, Zimmerberg J. Multiple local contact sites are induced by GPI-linked influenza hemagglutinin during hemifusion and flickering pore formation. Traffic. 2000;1:622-630.
8. Greengard O, Poltoratskaia N, Leikina E, Zimmerberg J, Moscona A. The anti-influenza virus agent 4-GU-DANA (zanamivir) inhibits cell fusion mediated by human parainfluenza virus and influenza virus HA. J Virol. 2000;74:11108-11114.
9. Huttner WB, Zimmerberg J. Implications of lipid microdomains for membrane curvature, budding and fission. Curr Opin Cell Biol. 2001;13:478-484.
10. Kim KM, Giedt CD, Basanez G, O'Neill JW, Hill JJ, Han YH, Tzung SP, Zimmerberg J, Hockenbery DM, Zhang KY. Biophysical characterization of recombinant human Bcl-2 and its interactions with an inhibitory ligand, antimycin A. Biochemistry. 2001;40:4911-4922.
11. Kuzmin PI, Zimmerberg J, Chizmadzhev YA, Cohen FS. A quantitative model for membrane fusion based on low-energy intermediates. Proc Natl Acad Sci USA. 2001;98:7235-7240.
12. Markovic I, Leikina E, Zhukovsky M, Zimmerberg J, Chernomordik L. Synchronized activation and unfolding of influenza virus hemagglutinins in multimeric fusion machine. J. Cell Biol. 2001;155:833-844.

13. Tzung SP, Kim KM, Basanez G, Giedt CD, Simon J, Zimmerberg J, Zhang KY, Hockenbery DM. Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. Nat Cell Biol. 2001;3:183-191.
14. Yergey AL, Coorssen JR, Backlund PS, Blank PS, Humphrey GA, Zimmerberg J, Campbell JM, Vestal ML. De novo sequencing of peptides using MALDI/TOF-TOF. J Am Soc Mass Spectrom. 2002;13:784-791.
15. Zimmerberg J. Are the curves in all the right places? Traffic. 2000;1:366-368.
16. Zimmerberg J. How can proteolipids be central players in membrane fusion? Trends Cell Biol. 2001;11:233-235.
17. Zimmerberg J, Blank PS, Kolosova I, Cho MS, Tahara M, Coorssen JR. A stage-specific preparation to study the Ca(2+)-triggered fusion steps of exocytosis: rationale and perspectives. Biochimie. 2000;82:303-314.

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# LATERAL INTERACTIONS BETWEEN ADJACENT FUSION PROTEINS: THE CASE OF INFLUENZA VIRUS HEMAGGLUTININ

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Membrane fusion is a common stage of diverse cell biological processes, including exocytosis, protein trafficking, fertilization, and entry of enveloped viruses into host cells. However, even for the best-characterized fusion process, which is mediated by influenza virus hemagglutinin (HA), the mechanism remains unknown. At the time of fusion, membranes are packed with fusogenic proteins, but we do not know whether each of these proteins serves as an independent fusion machine, whether adjacent individual proteins interact with each other in the plane of the membrane, or whether proteins outside the contact zone are involved in fusion. To address these questions experimentally and to characterize the role of HA-HA interactions, we investigated the effects of the surface density of HA on the efficiency of HA activation and fusion. Based on the results of these studies and an analysis of the literature, we have proposed a new mechanism of protein-mediated fusion.

## Synchronized Activation and Unfolding of Influenza Virus Hemagglutinins in Multimeric Fusion Machine

*Markovic, Leikina, Zhukovsky, Chernomordik; in collaboration with Zimmerberg*

Many authors have hypothesized that fusion is mediated by a multiprotein machine that somehow delivers the conformational energy of multiple proteins to the intermediates of membrane fusion. If this is correct, it is appropriate to ask how these proteins synchronize their refolding to minimize dissipation of the energy. We found that the proximity of other HAs affects triggering of the conformational change in an individual HA trimer. We modified the surface density of the HA of the Japan and X31 strains of influenza and assayed the transition of HA from its initial to its low pH conformation, measured as both the development of HA susceptibility to S-S reduction and the digestion of the exposed fusion peptide by thermolysin. Conformational change in HA was also detected functionally as inactivation of HA by low pH pretreatment in the absence of a target membrane. As expected, Japan HA–membranes retained fusogenic activity after longer low-pH incubations than did X31 HA–membranes. Our results suggest that the difference reflects slow activation rather than inactivation, as formerly thought. More important, we show that in both slow- and fast-activating strains, the percentage of activated HA increases with the increase in HA density, indicating that HA activation involves positive inter-trimer cooperativity. We propose that the spreading of the activation among adjacent HA trimers leads to the synchronized release of their conformational energy and is the mechanism by which multiple fusion proteins coordinate their activity at the fusion site (Markovic *et al.*, 2001).

### Reversible Stages of the Low-pH–Triggered Conformational Change in Influenza Virus Hemagglutinin

*Leikina, Ramos, Markovic, Chernomordik; in collaboration with Zimmerberg*

The refolding of the prototypic fusogenic protein hemagglutinin at the pH of fusion has often been thought of as a concerted and irreversible discharge of a loaded spring, with no distinct intermediates between the initial and final conformations. However, in our new study, we found that hemagglutinin refolding involves reversible conformations with a lifetime of minutes. After reneutralization, low pH–activated hemagglutinin returns from the conformations, wherein both the fusion peptide and the kinked loop of the HA2 subunit are exposed even though the HA1 subunits have not yet dissociated, to a structure that is functionally, biochemically, and immunologically indistinguishable from the initial structure. The rate of the transition from reversible conformations to irreversible refolding depends on pH and the presence of target membrane. Importantly, recovery of the initial conformation is blocked by the interactions between adjacent hemagglutinin trimers.

To explain the positive cooperativity of HA activation at low pH (see above), we hypothesized that individual HA trimers first establish a reversible, activated conformation. Our new work confirms our prediction experimentally and identifies an early reversible form of low pH–activated HA from which HA can revert to the initial conformation, if there are no adjacent trimers with which to interact. Inter-trimer interaction shifts HA restructuring toward irreversible stages. We propose that the existence of this relatively long-lived interme-diate state before the major refolding of HA is of importance for coupling between HA refolding and membrane fusion. We hypothesize that, at low pH, HA starts to flicker between its initial conformation and an early "primed" reversible state, with most of the time spent in the initial conformation. The delay before the discharge of most of the conformational energy of HA gives the adjacent activated trimers in the contact region time to interact and to synchronize their discharge.

While pathways of diverse membrane fusion reactions appear to have common membrane intermediates, the structures of the specialized fusion proteins can be rather dissimilar. However, reversible stages of refolding of activated fusion protein identified in our work for the fusion protein of influenza virus have been discussed in the literature for some other viruses, including tick-borne encephalitis, rabies virus, and HIV. By analogy with HA-mediated fusion, we hypothesize that different viral fusion reactions and intracellular fusion involve a distinct reversible stage of refolding of fusogenic proteins that allows adjacent trigger-activated proteins to assemble at the contact site. Subsequent concerted discharge of most of the conformational energy of these proteins drives membrane fusion (Leikina *et al.*, 2002).

### The Protein Coat in Membrane Fusion: Lessons from Fission

*Chernomordik; in collaboration with Kozlov*

Multiple cell biological processes involve two opposite rearrangements of membrane configuration referred to as fusion and fission. While membrane intermediates in protein-mediated fusion have been studied in some detail, the global force that drives sequential stages of fusion reaction, from early local intermediates to an expanding fusion pore, remains unknown. Neither of the published hypothetical mechanisms of protein-mediated fusion has addressed the question of how fusion proteins generate the membrane tension necessary for expansion of the fusion pore. A local protein machine is unable to generate tension in a membrane part larger than the initial fusion site. To generate a membrane stress that drives expansion of the fusion pore, the fusion machine must act on a large area of the membrane. This consideration, along with the established role of interactions between low-pH–activated HA in fusion, has motivated us to search for a mechanism that is based on fusion protein aggregation.

Fusion proceeds via stages that are analogous but oppositely directed to membrane budding-off and fission driven by protein coats. The energy needed for membrane budding and for fission of the membrane neck in the best-characterized budding-fission reactions, including intracellular fission and exit of enveloped viruses from host cells, is apparently produced by self-assembly of the coat proteins at the membrane surface. On the basis of the analogy between fusion and fission, we propose that an interconnected coat, formed by membrane-bound activated fusion proteins surrounding the membrane contact zone, generates the driving force for fusion. This fusion protein coat has a strongly curved intrinsic shape of opposite curvature to that of the protein coat driving fission. Since the rigidity of the protein coat is most likely much greater than that of the lipid bilayer, to adopt the intrinsic shape and thus to relieve the internal elastic stresses, the protein coat spontaneously bends out of the initial shape of the membrane surface. The bending produces elastic stresses in the underlying lipid bilayer and drives its fusion with the apposing membrane. The hypothesis that fusion proteins outside the contact zone participate in the generation of the driving force for fusion offers a new interpretation for a number of known features of the fusion reaction mediated by the prototype fusion protein, influenza hemagglutinin, and might bring new insights into mechanisms of other fusion reactions (Kozlov and Chernomordik, 2002).

## SELECTED PUBLICATIONS

1. Chernomordik LV, Melikyan GB. Membrane fusion and ten reasons not to study it. Biol Membr. 2001;18:475-486.
2. Epand RF, Yip CM, Chernomordik LV, LeDuc DL, Shin YK, Epand RM. Self-assembly of influenza hemagglutinin: studies of ectodomain aggregation by in situ atomic force microscopy. Biochim Biophys Acta. 2001;1513:167-175.
3. Greengard O, Poltoratskaia N, Leikina E, Zimmerberg J, Moscona A. The anti-influenza virus ahent 4-GU-DANA (Zanamivir) inhibits cell fusion mediated by human parainfluenza virus and influenza virus HA. J Virol. 2000;74:11108-11114.
4. Kozlov MM, Chernomordik LV. The protein coat in membrane fusion: lessons from fission. Traffic. 2002;3:256-267.
5. Kozlovsky Y, Chernomordik LV, Kozlov MM. Lipid intermediates in membrane fusion: formation, structure, and decay of hemifusion diaphragm. Biophys J. 2002;83:2634-2651.
6. Leikina E, Chernomordik LV. Reversible merger of membranes at the early stage of influenza hemagglutinin-mediated fusion. Mol Biol Cell. 2000;11:2359-2371.
7. Leikina E, LeDuc DL, Macosko JC, Epand R, Epand R, Shin YK, Chernomordik LV. The 1-127 HA2 construct of influenza virus hemagglutinin induces cell-cell hemifusion. Biochemistry. 2001;40:8378-8386.
8. Leikina E, Markovic I, Chernomordik LV, Kozlov MM. Delay of influenza hemagglutinin refolding into a fusion-competent conformation by receptor binding: a hypothesis. Biophys J. 2000;79:1415-1427.
9. Leikina E, Mertts MV, Kuznetsova N, Leikin S. Type I collagen is thermally unstable at body temperature. Proc Natl Acad Sci USA. 2002;99:1314-1318.
10. Leikina E, Ramos C, Markovic I, Zimmerberg J, Chernomordik LV. Reversible stages of the low-pH-triggered conformational change in influenza virus hemagglutinin. EMBO J. 2002;21:5701-5710.
11. Markovic I, Leikina E, Zhukovsky M, Zimmerberg J, Chernomordik LV. Synchronized activation and unfolding of influenza virus hemagglutinins in multimeric fusion machine. J Cell Biol. 2001;155:833-844.
12. Melikov KC, Frolov VA, Shcherbakov A, Samsonov AV, Chizmadzhev YA, Chernomordik LV. Voltage-induced non-conductive pre-pores and metastable single pores in unmodified planar lipid bilayer. Biophys J. 2001;80:1829-1836.
13. Mittal A, Leikina E, Bentz J, Chernomordik LV. Kinetics of influenza hemagglutinin mediated membrane fusion as a function of technique. Anal Biochem. 2002;303:145-152.

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# ADVANCES IN THE METHODOLOGY OF ELECTROPHORETIC SEPARATIONS

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Our work aims at increasing the effectiveness of electrophoretic separations of biological macromolecules and subcellular particle-sized species by application of separation theory through development of computer programs and by instrumental and procedural optimization at a level that is practical for the vast majority of biochemical laboratories. We continued to work on improving the method of “direct electroelution” of proteins from polyacrylamide gels and that of “static noncovalent” coating of capillaries for reducing electroosmotic flow in capillary electrophoresis (CE) to a negligible value. Using rat synaptic vesicles as a model, we have been attempting to develop a novel approach to deciphering protein complexes based on their cross-linking and fractionation by capillary electrophoresis. The complexes are to be continuously blotted to membrane, identified as SNARE protein complexes, followed by a mass-spectrometric (MS) characterization of interacting proteins. We have also been attempting to improve the resolution at low gel concentration of fluorescently labeled saccharides by using acetate as the trailing constituent of a disc electrophoresis buffer system and to replace 2D-PAGE in proteomic analysis of a representative membrane protein by a three-stage procedure of SDS-PAGE, electroelution, IPG-IEF, and band transfer into MS.

## Direct Electroelution of Protein Bands from SDS-PAGE

*Radko, Zakharov, Chrambach; in collaboration with Chang, Bezrukov, Yergey, Vieira*

We further developed direct protein elution from SDS-PAGE bands without gel sectioning to a sensitivity toward 0.1-0.2 μg of protein through use of a green laser to detect the SYPRO-red labeled protein, providing a five-fold increase in sensitivity over that previously achieved by sideways UV illumination of the band. Electroelution time was inversely proportional to protein mobility; at 1 kV, it ranged from 1 to 12 minutes for various proteins to attain 80 percent or more electroelution from 1-mm-thick Novex gels. After three steps of ultrafiltration in water to deplete SDS, protein yield decreased to 25 to 33 percent for various proteins. To obtain a signal/noise ratio of six, a load of 2 picomoles of protein on SDS-PAGE was required. Instrument developments included replacement of the wick between electroelution tube and anolyte by direct connection of a large anolyte reservoir to the tube and a scaled-down procedure that, in lieu of the electroelution tube, employs a capillary of a custom-made CE apparatus equipped with a laser-induced fluorescence (LIF) detector. In addition to the transfer of electroeluate into MS, we demonstrated the transfer onto immobilized pH gradient/iso-electric focusing (IPG-IEF) strips.

### Resolving Capacity of Capillary Electrophoresis as a Function of the Hydrophilicity of Adsorbed Static Coating

*Chrambach; in collaboration with Cretich, Chiari, Stastna*

To obviate electroosmotic flow, capillaries in CE are conventionally covalently coated internally with polyacrylamide. We were able to show previously that the noncovalent coating with epoxy-poly-dimethylacrylamide (EPDMA) is a labor- and time-saving alternative to that procedure. However, EPDMA coating is unstable at very low ionic strength and in the presence of SDS, suggesting a hydrophilic binding between the coating and the silanol of the inner capillary wall. Thus, we attempted to improve the coating by derivatizing EPDMA so as to make it more hydrophilic. We demonstrated that a moderate degree of such derivatization indeed gave rise to less protein adsorption onto the capillary wall (decreased peak asymmetry) and increased resolving capacity (decreased peak width). Excessive hydrophilicity, however, led to electrostatic repulsion between silanol and coating and therefore lability of the coating.

### Deciphering Protein Complexes: Search for Rat Synaptic Vesicle Proteins Participating in Membrane Fusion

*Radko; in collaboration with Blank, Zimmerberg*

Over the last years, accumulated observations have indicated that Ca-dependent membrane fusion cannot be accounted for by the action of SNARE proteins alone; instead, fusion appears to depend on hitherto other unknown fusigenic proteins. We initiated a search for those proteins by using the small but abundantly available rat synaptic vesicle as a membrane preparation. We planned to develop a new approach to separation of large protein complexes that are obtained by treating synaptic vesicles with various cross-linking agents under conditions promoting vesicle fusion, followed by solubilization of the vesicles. The approach is based on capillary electrofocusing and zone electrophoresis of cross-linked complexes, combined with direct continuous elution and blotting of the separated peaks onto a membrane by means of electro-osmotic flow. The material collected on a blotting membrane will then be tested immunologically for the presence of a SNARE protein. After dissociation of the cross-links and/or digestion with trypsin, the immunoactive spot(s) should allow for the mass spectrometric identification of the SNARE-associated proteins. As a first step, we isolated rat synaptic vesicles by employing differential velocity centrifugation, followed by controlled pore glass (CPG) chromatography. We investigated the degree of homogeneity of the vesicles by capillary zone electrophoresis (CZE). The vesicle preparations before and after velocity sedimentation in sucrose gradients and those after CPG chromatography all exhibit distinguishable CZE peaks, suggesting distinct surface charge densities depending on particle size. We have found that the following CZE conditions reveal size differences: 0.02M polyethylene oxide ($M_r$ 4 million) in the CZE buffer and a field strength of 30 kV. We are also searching for the *in vitro* conditions to promote Ca-dependent fusion (within physiological range of calcium concentrations) of the vesicles either in the homotypic manner or to target membranes such as liposomes.

### Enhanced Gel Electrophoretic Resolution by Application of an Extensive Computer Output of Discontinuous Buffer Systems

*Chrambach; in collaboration with Cabanes-Macheteau, Taverna, Berna, Ashburn, Wheeler*

We achieved gel electrophoretic separation of small, highly charged saccharides in discontinuous buffer systems at low gel concentration (less than 5 percent T) by selecting acetate as the trailing constituent of the moving boundary “front” in lieu of the conventionally used glycinate or borate (Fig. 14). That selection was based on the use of the output of a computer program of T. M. Jovin, Max Planck Institut für Biophysikalische Chemie, Göttingen, Germany, which was hitherto restricted to the NIH mainframe computer. To make this output and its benefits, as demonstrated by the improved resolution of the representative saccharide separation problem, more widely available, we made the discontinuous buffer systems across the entire pH scale, 0 and 25C, available on the Internet (http://www.ncbi.nlm.nih.gov/Class/wheeler/jovin.html).

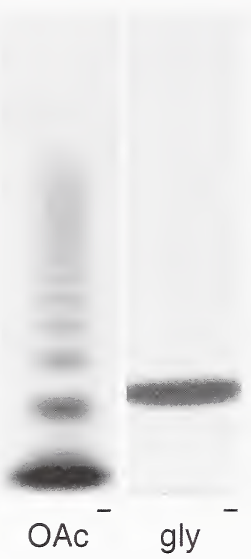

**FIGURE 14**

Separation by PAGE of ANTS-labeled dextran homopolymers as a function of the trailing constituent of a discontinuous buffer system. The trailing buffer constituent is, left: acetate; right: glycinate. All other buffer constituents (leading constituent chloride; common constituent Tris) and the gel concentration (4.5 %T, 2.7 $\%C_{Bis}$) are the same in both cases. The operative pH values are 8.78 and 9.4, 25°C, the ionic strength 0.0793 and 0.0153 for the acetate and glycinate systems, respectively. ANTS = 8-aminonaphthalene-1,3,6-trisulfonic acid.

### Three-Stage Electrophoretic Procedure Replacing 2D-PAGE in the Proteomics of Membrane Proteins

*Buzas, Radko, Chrambach; in collaboration with Sarkadi*

Proteomics is burdened by the frequent inapplicability of conventional 2-D PAGE to membrane proteins, which constitute one half of cellular proteins. The inapplicability is attributable to the membranes' limited solubility in detergents compatible with first-dimensional IPG-IEF. Moreover, effective proteomic analysis is burdened by the need for multiple 2D gels with first-dimensional IEF at multiple narrow pH gradients. Both problems can be solved by replacing 2-D-PAGE with a three-stage procedure (3S-PAGE) in which SDS-PAGE is carried out as a first stage, followed by direct electroelution of the component of interest and analysis of the electroeluate by simultaneous IEF on IPG-strips of the desired pH ranges. Separated protein bands are then transferred into mass spectrometry by conventional proteomic procedures. We implemented 3S-PAGE using the membrane protein MRP1. The crude extract containing the protein was solubilized in media consisting of ASB-16, 7 M urea, 2 M thiourea, or of 4 percent CHAPS, 8 M urea. However, MRP1 immunologically detected in the latter solution fails entirely to enter into IPG-IEF while MRP1 from the former solution falls into two fractions, the major one of which fails to migrate in the electrofocusing gels. Those separation problems show MRP1 to be a representative candidate for development of the 3S-PAGE procedure, which is presently under way.

## SELECTED PUBLICATIONS

1. Buzas Zs, Chang H-T, Vieira, NE, Yergey AL, Stastna M, Chrambach A. Direct vertical electroelution of protein from a PhastSystem band for mass spectrometric identification at the level of a few picomoles. Proteomics. 2001;1:691-698.
2. Buzas Zs, Li T, Chrambach A. Horizontal gel electrophoresis of SDS-proteins on the PhastSystem with an at least twenty five-fold increased protein load volume. Anal Biochem. 2001;292:161-163.
3. Chang HT, Yergey AL, Chrambach A. Electroelution of proteins from bands in gel electropherograms without gel sectioning for the purpose of protein transfer into mass spectrometry: elements of a new procedure. Electrophoresis. 2001;22:394-398.
4. Chiari M, Cretich M, Stastna M, Radko SP, Chrambach A. Rapid capillary coating by epoxy-poly(dimethylacrylamide). Electrophoresis. 2001;22:656-659.
5. Chrambach A. Gel electrophoresis: one-dimensional. In: Encyclopedia of life sciences. Vol.7. London: Macmillan Reference Ltd., 2002;579-581.
6. Chrambach A. Stagnation and regression concomitant with the advances in electrophoretic separation science. In: Issaq HJ, ed. A century of separation science. New York: Marcel Dekker, 2002;721-728.

7. Cretich M, Stastna M, Chrambach A, Chiari M. Decreased protein peak asymmetry and width due to static capillary coating with hydrophilic derivatives of poly(DMA). Electrophoresis. 2002;23:2274-2278.
8. Li YM, Chrambach A. Gel electrophoretic isolation in the hundred microgram range of recombinant SDS-syntaxin from sea urchin egg cortical vesicles. Prep Biochem Biotechnol. 2001;31:369-387.
9. Radko SP, Chang HT, Zakharov SF, Bezrukov L, Yergey AL, Vieira NE, Chrambach A. Direct electroelution of proteins from SDS-PAGE for transfer into mass spectrometry: fluorescent detection and load of protein, recovery, re-analysis by IEF and MALDI-TOF of protein and tryptic peptides. Electrophoresis. 2002;23:985-992.
10. Radko SP, Chrambach A. Separation and characterization of submicron- and micron-sized particles by capillary zone electrophoresis. Electrophoresis. 2002;23:1957-1972.
11. Radko SP, Stastna M, Chrambach A. Capillary zone electrophoresis of submicron-sized particles in electrolyte solution of various ionic strengths: size-dependent electrophoretic migration and separation efficiency. Electrophoresis. 2000;21:3583-3592.
12. Radko SP, Stastna M, Chrambach A. Relation of peak width to polydispersity of liposome preparations subjected to capillary zone electrophoresis. J Chromatogr B. 2001;761:69-75.
13. Stastna M, Radko SP, Chrambach A. Discrimination between peak spreading in CZE of proteins due to interaction with the capillary wall and that due to protein microheterogeneity. Electrophoresis. 2001;22:66-70.
14. Yefimov S, Sjomeling C, Yergey AL, Chrambach A. Stacking of unlabeled SDS-proteins within a fluorimetrically detected moving boundary, electroelution and mass spectrometric identification. Electrophoresis. 2001;22:999-1003.
15. Yefimov S, Yergey AL, Chrambach A. Sequential electroelution and mass spectroscopic identification of intact SDS-proteins labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester. Electrophoresis. 2001;22:2881-2887.

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# APPLICATIONS OF MASS SPECTROMETRY TO BIOPHYSICS, BIOCHEMISTRY, AND MEDICINE

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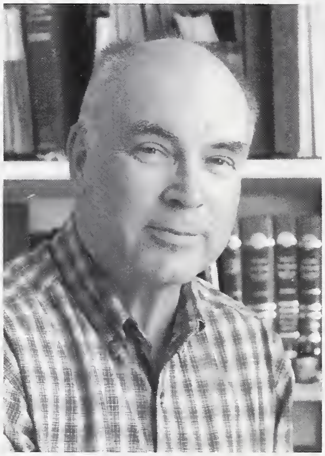

The section carries out research in areas of chemistry, biochemistry, and medicine in which mass spectrometry is the primary analytical tool. Current research focuses on the energetics of hydration, protein characterization, and the quantification of endogenous molecules.

## Hydration Thermodynamics

*Vieira, Gilligan, Yergey*

We are determining the energy required for noncovalent bonds between molecules and ions in solution to form and break. The particular bonds of interest are those involved in the interplay of solutes, such as amino acids, small peptides, and lipids, with water. The objective of our thermodynamic investigations is to determine solvation enthalpies and free energies of selected biologically interesting ions. Knowledge of the energetic requirements of these noncovalent bonds, particularly those involving water and biologically significant molecules, is fundamental to understanding molecular interactions and the changes in conformations that are integral to them.

Experimentally, we use the tools of equilibrium gas phase ion-molecule chemistry implemented in a modified electrospray ionization (ESI) source of a single-quadrupole mass filter. In this work, we determine the thermodynamic quantities deltaH(std)298, deltaS(std)298, and deltaG(std)298 by using the approach of equilibrium ion-molecule reaction chemistry. Hydration thermodynamics values are calculated from equilibrium constants measured over a temperature range of 0 to136 C° at ion source water partial pressures ranging from between zero and 100 mtorr. Equilibrium ion intensity measurements were made for at least four hydration states, i.e., zero through three water molecules associated with a core ion, and include at least 60 combinations of water partial pressures and temperatures covering the ranges of experimental variables. An exhaustive study of the equilibrium clustering of one to three waters around n-alkylammonium, CnH(2n+1)NH3+, and the di- and tri-methyl ammonium ions has shown a pattern of behavior consistent with water clusters forming around the charged portion of the ion and being influenced by the nature of the attached hydrophobic groups. Detailed analysis of the entropy values derived from the measurements is consistent with detecting the entropy change associated with the formation of an internal hydrogen bond.

Both the concept of water organizing around a charge site and the formation of internal hydrogen bonds detected by substantial entropy decreases have undergone further exploration with the investigation of the hydration energetics of simple alkyl-diols: 1,2-(OH)2-propane, 1,3-(OH)2-propane, 1,3-(OH)2- butane, and 1,4-(OH)2-butane. The stepwise addition of water to protonated 1,2-(OH)2-propane shows a trend of diminishing exothermicity for each addition. While we were unable to obtain a direct measurement for the addition of the first water, we were able to estimate an upper limit on the exothermicity for the same process based on the water partial pressure and temperature. We conclude that this first hydration step is energetically very favorable. We observed the decreasing trend in energetics for 1,2-(OH)2-propane for the addition of the first two water molecules to 1,3-(OH)2-propane but noted that the decrease was not maintained for the addition of the third water molecule. The addition of the third water to the complex was determined to be energetically more favorable than the addition of the second; in addition, we found a substantial decrease in the entropy for the process. These two observations led us to conclude that the 1,3-(OH)2-propane trihydrate is an energetically and entropically favorable state. The existence of such favorable hydration states is consistent with the observation that the 1,3-diol incorporates itself into several biologically interesting systems and disrupts otherwise stable biomolecular structures. On the other hand, both the butane diols behave in a manner consistent with the 1,2-propane diol.

Recent results from studies of the hydration of several amino acids show great promise for uncovering further structural information about the nature of water clusters and the concept of hydrophobicity. Results from the equilibrium clustering of water with several protonated neutral amino acids (glycine, valine, and leucine) show behavior that is similar to that observed with the alkylammonium ions for the first two water molecules. The addition of the third water, however, is associated with a decrease in both enthalpy and entropy, suggesting the formation of intramolecular hydrogen bonds as seen in the 1,3-propane diol. On the other hand, the results obtained to date in studies of the basic amino acids, arginine and lysine, are almost counterintuitive. That is, while we might expect the energetics of adding the first water to these molecules to be highly favorable, we have determined that it is less energetically favorable than the addition of the first water to the neutral amino acids, suggesting the presence of internal hydrogen bonding before any water clusters are formed.

### Protein Characterization

*Backlund, Gilligan, Tran, Vieira, Yergey; in collaboration with Blank, Humphrey, Zimmerberg, Crouch, DePamphilis, Epstein, Hinnebusch, Poszgay, Robbins, Rouault, Sackett, Schneerson, Leppla, Levine, Schuck, Alkon, Campbell, Coorssen, Hochberg, Vestal*

We conduct research on the mass spectrometric characterization of proteins. We carry out work collaboratively with groups in NICHD as our first priority but also conduct independent investigations. A major aspect of our work is the identification of proteins isolated in biochemical investigations.

For identification of unknown proteins, we use mass spectrometric data to query genomic databases to answer the general question of whether any of the protein sequences present in the databases have expected proteolytic cleavage products with theoretical masses that match the empirically determined masses of the peptides generated from the unknown. Three mass spectrometric approaches are available for this effort: Matrix Assisted Laser Desorption Ionization (MALDI) with Time-of-Flight (TOF) mass analysis; liquid chromatography (LC) followed by electrospray ionization with mass analysis in an instrument capable of using fragmentation reactions to generate peptide sequences (LC-MS/MS); and, most recently, MALDI followed by tandem TOF analysis for the determination of peptide sequences from fragment ion spectra. The last, state-of-the-art technology has become available during the year and has been demonstrated to provide identifications of mixtures of proteins at levels of about 100 fmole. With the current combination of instrumentation, we are confident that, given enough material in a gel band to make 100 fmole available for analysis, we can positively identify a protein that is described in a database.

We are pursuing two development projects designed to improve protein characterization capabilities. First, we have begun addressing the issue of providing sequence information on proteins that are not described in databases because the database either is incomplete or contains errors. We are taking the approach termed complete *de novo* sequencing of peptides. That approach, which requires detailed interpretation of individual fragmentation mass spectra of peptides, is being implemented in conjunction with software designed and written in our section. To date, we have applied the approach to the sequencing of peptides from a series of standard tryptic digests as well as to tryptic digests of proteins isolated from sea urchin cortical vesicles. Our results have shown that not only does the program identify from a spectrum the correct sequences with the highest-scoring possibility but that the spectra allow Leu residues to be distinguished from Ile. In a related area, we have made substantial progress in the development of a consistent methodology for the identification of site-specific phosphorylation. A combination of collision-induced fragmentation (CID) under LC-MS/MS conditions to identify single phosphorylations and thereby follow neutral phosphate losses with identification of multiple phosphorylations on single peptides under non–CID conditions with the MALDI TOF/TOF appears to hold considerable promise.

### Ratio of Endogenous Cortisone to Cortisol

*Li, Yergey; in collaboration with Chrousos, Hochberg, Zoumakis*

The purpose of this work is to use mass spectrometry to assess the activity of 11-hydroxysteroid dehydrogenase (HSD) types I and II in both cell culture and clinical studies. HSD-I has recently been shown to contribute to a hormonally dependent obesity when the enzyme is up-regulated in peripheral fatty tissue. The obesity is a consequence of conversion of metabolically inactive cortisone into cortisol through the reduction of a double bond in the steroid by HSD-I. On the other hand, HSD-II is responsible for the reverse conversion. It is hypothesized that increased activity of 11-HSD-I will be recognizable by increased levels of urinary cortisol excretion. As part of a potential therapeutic approach to treating this disorder, an evaluation of 11-HSD-I activity in cultured fibroblasts under conditions of exposure to a variety of candidate agonists is also under way. Experimentally, we have developed an electrospray ionization LC-MS method that is capable of determining the ratio of cortisone to cortisol in both urine and culture media over a 100-fold dynamic range at picomole levels of material injected on column. Preliminary results of these studies have been satisfactory for the purposes of analysis of tissue culture media and for the determination of a suite of urinary steroids.

## SELECTED PUBLICATIONS

1. Blank PS, Sjomeling CM, Backlund PS, Yergey AL. Use of cumulative distribution functions to characterize mass spectra of intact proteins. J Am Soc Mass Spectrom. 2002;13:40-46.
2. Gilligan JJ, Schuck P, Yergey AL. Mass spectrometry after capture and small-volume elution of analyte from a surface plasmon resonance biosensor. Anal Chem. 2002;74:2041-2047.
3. Nelson TJ, Backlund PS, Yergey AL, Alkon DL. Systematic identification of protein-protein interactions by mass spectrometry. Molec Cell Proteomics. 2002;1:253-259.
4. Pozsgay V, Vieira NE, Yergey AL. A method for bioconjugation of carbohydrates using diels-alder cycloaddition. Org Lett. 2002;4:3191-3194.
5. Praetorius J, Backlund P, Yergey AL, Spring KR. Specific lectin binding to beta-1 integrin and fibronectin on the apical membrane of Madin-Darby canine kidney cells. J Membr Biol. 2001;184:273-281.
6. Yergey AL, Campbell JM, Coorssen JR, Backlund PS, Blank PS, Humphrey GA, Zimmerberg J, Vestal ML. *de novo* sequencing of peptides using MALDI TOF-TOF. J Am Soc Mass Spectrom. 2002;13:784-791.

## COLLABORATORS

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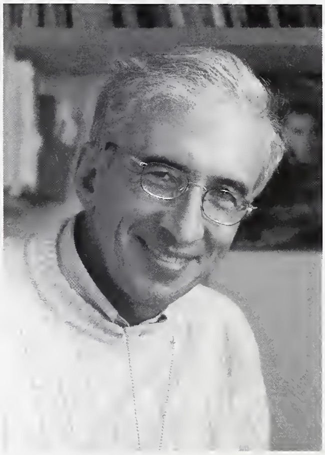
# INTERACTIONS OF HUMAN PATHOGENS IN HUMAN LYMPHOID TISSUE



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Critical events of HIV disease occur in lymphoid tissue. One such event is HIV-1 divergence into a "swarm" of viral quasi-species whose dominant forms change in the course of the disease. In particular, CCR5-utilizing (R5) HIV-1 variants predominate in the early stages of HIV-1 disease, whereas CXCR4-utilizing (X4) HIV-1 variants may dominate later. When such a switch occurs, it is followed by an acceleration of disease progression to AIDS. The general aim of this project is to investigate the mechanisms underlying the R5-to-X4 switch and the immunodeficiency development associated with such a switch. We postulate that R5 and X4 HIV variants affect each other and that various microbes can selectively inhibit or stimulate R5 or X4 replication by modulating the chemokine/cytokine network, thus contributing to the switch of viral dominance. We addressed mechanisms of HIV-1 immunopathogenesis in human lymphoid tissue *ex vivo*, tissue that preserves tissue cytoarchitecture and supports productive HIV infection without exogenous stimulation or activation. These tissues are infected *ex vivo* with a mixture of HIV-1 quasi-species differing in co-receptor usage and with HIV co-pathogens, in particular human herpesvirus 6 (HHV-6).

## CXCR4-Tropic HIV-1 Suppression of CCR5-Tropic HIV-1 Replication in Human Lymphoid Tissue *ex Vivo* by Selective Induction of CC-Chemokines

*Ito, Grivel, Chen, Kiselyeva, Margolis; in collaboration with Reichelderfer*

To evaluate replication of individual CCR5- and CXCR4-utilizing variants, we developed a new real-time RT PCR–based assay that accurately estimates the number of viral-specific RNA copies even when their relative abundance is two orders of magnitude. We designed primers that recognize only target sequences of gp120 that are specific for R5 and X4 HIV-1 variants and that do not give any non-specific amplification. We further improved the assay so that variations were small and the standard curves were linear, allowing a reliable determination of the number of RNA copies in experimental samples. We verified the accuracy of the assay by comparing the results obtained with viral-specific primers and those obtained with a primer common to all the viruses. Moreover, the amounts of HIV-1 RNA copies in various experimental conditions correlated with the concentrations of p24, another conventional assay to determine virus replication. We applied the newly developed assay to an *ex vivo* model consisting of blocks of human lymphoid tissue infected with mixtures of R5 and X4 HIV-1 variants.

To study whether the R5 and X4 viruses interfere with each other in the context of lymphoid tissue, we used two pairs of HIV-1, one pair consisting of the prototypic X4 and R5 HIV-1 variants, LAV.04 and SF162, respectively, and the other pair consisting of the "X4-skewed" dual-topic (R5X4) 89.6 and its R5 chimera 89-v345.SF. On average, LAV.04 infection suppressed SF162 replication to about 7 percent of the level of tissues infected with SF162 alone. In contrast, the levels of LAV.04 replication in tissues co-infected with SF162 did not differ from those found in controls infected only with LAV.04. Further, 89.6 replicated in human lymphoid tissue to a level similar to that found in singly infected tissue, whereas replication of the R5-chimera 89-v345.SF in tissues co-infected with 89.6 was suppressed about five-fold compared with matched tissues infected with 89-v345SF only. To study whether X4 merely prevents the establishment of R5 infection or affects ongoing R5 infection as well, we delayed tissue inoculation with LAV.04 for 3, 24, 72, 96, or 144 hours to allow the establishment of SF162 infection. In all experiments, LAV.04 significantly inhibited SF162 replication in co-infected tissues compared with singly infected tissues.

Given that CC-chemokines are potent inhibitors of replication of R5 HIV-1 variants in both isolated cells and human lymphoid tissues, we investigated whether X4 infection up-regulates CC-chemokines. Using a multiplex Luminex assay, we evaluated the concentrations of a panel of cytokines in the culture medium bathing the lymphoid tissue. In SF162-infected tissues, the average concentrations of all measured chemokines/cytokines were similar to those in uninfected controls. In contrast, in tissues infected with LAV.04 either alone or together with SF162, the levels of MIP-1$\alpha$, MIP-1$\beta$, and RANTES increased four- to seven-fold compared with those in matched uninfected controls. Flow cytometry revealed that X4 infection augments both the number of chemokine-producing cells and cell productivity. A cocktail of exogenous CC-chemokines at concentrations similar to those induced by LAV.04 simulated the effects of the X4 virus on SF162 replication.

We conclude that X4 and R5 HIV-1 variants interfere with each other in human lymphoid tissues and that such interference is associated with an up-regulation of CC-chemokines. X4 inhibition of R5 replication may result in a shift of dominance among the HIV-1 variants, thus contributing to the R5-X4 switch.

### Selective Suppression of CCR5 HIV-1 Variants by Co-Infection with HHV-6 in Human Lymphoid Tissue *ex Vivo*

*Grivel, Ito, Fitzgerald, Margolis; in collaboration with Fagà, Lusso, Malnati, Santoro*

*In vivo*, HIV disease progression is associated with the emergence of concurrent infections that may affect the course of the disease by mechanisms that remain unknown. A lymphotropic agent frequently reactivated in HIV-infected patients is human herpesvirus 6 (HHV-6), which has been proposed as a co-factor in AIDS progression. As multiple interactions have been documented between HHV-6 and HIV-1 both *in vitro* and *in vivo*, we investigated whether the two viruses affect each other within the context of human lymphoid tissue.

We developed a new model system that uses human lymphoid tissue cultured *ex vivo* to study the cellular tropism and pathogenic mechanisms of HHV-6. Viral strains belonging to both subgroup A (strain GS) and subgroup B (strain PL1) were able to infect human tonsillar tissue fragments productively. The majority of productively infected cells were CD4$^+$ T lymphocytes expressing a memory phenotype (CD45RA$^-$62L$^-$) while infection of CD8$^+$ T cells was efficient only with HHV-6 A. Little, if any, HHV-6 replication was documented in B cells and non-lymphoid cells. Expression of CD4 was up-regulated, whereas both CD46, which serves as a cellular receptor for HHV-6, and CD3 were down-regulated. The results provide a novel *ex vivo* model for investigating the complex interactions between HHV-6 and other pathogens of the immune system, in particular HIV.

Strikingly, co-infection with HHV-6 differentially affected the replication of the R5 and X4 HIV-1 variants. A dramatic (five-fold) suppression of HIV-1 replication was observed in tissues co-infected with HHV-6 and the CCR5-using variant SF162. In contrast, we noted a slight enhancement in the replication of the CXCR4-using variant LAV.04 in matched tissue blocks.

To prove conclusively that the differential effect of HHV-6 on HIV-1 replication was related to HIV-1 co-receptor tropism, we used two isogenic dual-tropic molecular clones, 89.6 and 89-v345.SF, described above, that behave in *ex vivo* human lymphoid tissue as CXCR4- or CCR5-tropic variants. In agreement with our results with uncloned HIV-1 isolates, HHV-6 co-infection of lymphoid tissue resulted in a marked (five-fold) inhibition of 89-v345.SF replication but did not affect 89.6 replication. To investigate the potential mechanisms underlying the effects of HHV-6 on HIV-1 infection, we evaluated the production of RANTES, MIP-1a, and MIP-1b, the three CC chemokines that bind to CCR5, as they were reported to cause similar effects on the replication of different HIV-1 strains. In all the tissues infected with HHV-6, either alone or in combination with HIV-1, we documented a dramatic increase in the production of RANTES, whereas the levels of MIP-1a and MIP-1b were not significantly augmented. Regression analysis demonstrated a significant correlation between HHV-6 replication and RANTES up-regulation. Moreover, we observed a significant correlation between HHV-6–induced RANTES up-regulation and suppression of SF162 replication. Flow cytometry of cells isolated from HHV-6–infected tissue blocks also documented increased numbers of RANTES-producing cells; interestingly, about one-third of the RANTES-producing cells were infected by HHV-6. Adding RANTES to the culture medium in amounts similar to those induced by HHV-6 causes inhibition of R5, but not X4 replication, to a level similar to that caused by HHHV-6 co-infection.

Our results provide the first demonstration that another viral agent can selectively affect HIV-1 infection in the context of human lymphoid tissue. Our observations suggest that HHV-6 replication, an event that frequently occurs *in vivo* during the progression of HIV-1 disease, selectively suppresses CCR5-utilizing HIV-1 variants while not affecting or even stimulating the replication of CXCR4-tropic variants. Thus, it is possible that HHV-6 favors the latter variants in co-infected tissues, from which they can spread throughout the entire organism and become dominant, as frequently occurs during AIDS progression. Exploitation of the ability of HHV-6 to induce RANTES in lymphoid tissue might lead to novel therapeutic and preventive strategies against HIV infection.

## SELECTED PUBLICATIONS

1. Glushakova S, Munch J, Carl S, Greenough TC, Sullivan JL, Margolis L, Kirchhoff F. CD4 down-modulation by human immunodeficiency virus type 1 nef correlates with the efficiency of viral replication and with CD4(+) T-cell depletion in human lymphoid tissue ex vivo. J Virol. 2001;75:10113-10117.
2. Gondois-Rey F, Grivel JC, Biancotto A, Pion M, Vigne R, Margolis L, Hirsch I. Segregation of R5 and X4 HIV-1 variants to memory T cell subsets differentially expressing CD62L in ex vivo infected human lymphoid tissue. AIDS. 2002;16:1245-1249.
3. Grivel JC, Ito Y, Fagá G, Santoro F, Shaheen F, Malnati M, Fitzgerald W, Lusso P, Margolis L. Suppression of CCR5- but not CXCR4-tropic HIV-1 in lymphoid tissue by human herpesvirus 6. Nat Med. 2001;7:1232-1235.
4. Malkevitch N, McDermott DH, Yi Y, Grivel JC, Schols D, De Clercq E, Murphy PM, Glushakova S, Collman R, Margolis L. Coreceptor choice and T cell depletion by R5, X4 and R5X4 HIV-1 variants in CCR5-deficient (CCR5Δ32) and normal human lymphoid tissue. Virology. 2001;281:239-247.
5. Malkevich N, Womack C, Pandya P, Grivel JC, Fauci AS, Margolis L. Human immunodeficiency virus type 1 (HIV-1) non-B subtypes are similar to HIV-1 subtype B in that coreceptor specificity is a determinant of cytopathicity in human lymphoid tissue infected ex vivo. J Virol. 2001;75:10520-10522.

## COLLABORATORS

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# STRUCTURE AND FUNCTION OF GLUTAMATE RECEPTORS

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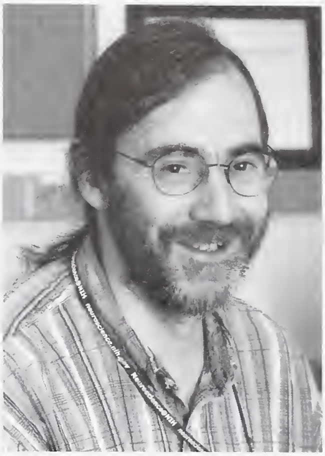

Ionotropic glutamate receptors (GluRs) are molecular pores that facilitate the passage of ions across cell membranes. The mechanism mediates excitatory signal transmission in the mammalian nervous system at the majority of synapses. Given the receptors' essential role in normal brain function and development and increasing evidence that dysfunction of GluR activity underlies multiple diseases of the central nervous system as well as damage during stroke, we have directed much effort toward analyzing GluR function at the molecular level. A major goal of our work is to obtain structural data that will allow the development of subtype-selective antagonists and allosteric modulators. In humans, the ionotropic glutamate receptors are encoded by seven gene families named after the ligands that were first used to identify the major subtypes on a functional basis: AMPA, kainate, and NMDA. In addition, a growing family of prokaryotic GluRs is emerging from the sequencing of microbial genomes, of which GluR0 from the photosynthetic bacterium syncheocystis PCC 6803 was the first to be identified. Much work remains to be done, including studies on other subtypes and domains that were excluded from the constructs used in our initial studies.

## Expression of Kainate Receptor Ligand-Binding Cores

*Mayer*

The recent crystallization of the ligand-binding cores of an AMPA receptor subunit and GluR0 has revealed, for the first time, the molecular mechanisms underlying the binding of agonists and antagonists. By studying the structural and functional changes produced by mutations that alter the gating of ion channel activity of the GluR2 AMPA receptor subunit, we have obtained evidence that defines a major structural role for an interface between the ligand-binding cores of individual subunits. Our studies show that, for ion channel gating to proceed, the subunits must assemble as dimers. Drugs or mutations that increase dimer stability attenuate desensitization and facilitate gating while mutations that reduce dimer stability enhance desensitization. Our studies reveal, for the first time, a molecular mechanism for desensitization of a ligand-gated ion channel.

Ongoing work in the laboratory has focused on the establishment of procedures for the expression, biophysical characterization, and crystallization of the ligand-binding cores of kainate subtype GluRs. Our goal is to characterize members of both the high- (KA1 / KA2) and low- (GluR5-GluR7) affinity kainate receptor gene families. Based on the design of the previously characterized GluR2 and GluR0 ligand-binding cores, we assembled related constructs for GluR5, GluR6, KA1, and KA2 for expression in *E. coli.* At present, procedures have been established for the production of highly purified GluR6 at the 20 mg scale and of GluR5 at the 5 to 10 mg scale. Both proteins were expressed in the cytoplasm of *E. coli* with N-terminal His tags and purified by NTA affinity chromatography followed by proteolytic removal of the His tag and final purification by ion exchange chromatography. The proteins are monodisperse when concentrated to 20 mg/ml and have the expected molecular weight when analyzed by electrospray mass spectrometry. We have grown crystals of a GluR6 complex with L-glutamate and characterized them by x-ray diffraction. The lattice is orthorhombic with unit cell dimensions a = 57.8 A, b= 90.8 A, c= 104.8 A. We have tentatively identified the space group as P21212, and diffraction to < 2.2 A is obtained on a rotating anode x-ray source. With the crystals not sufficiently well ordered for a high-resolution structure determination, crystallization conditions are undergoing optimization to address this deficiency. Additional agonists for which we intend to solve GluR6 complexes include kainate, quisqualate, and 4-methly-glutamate, a high-affinity kainate receptor agonist. Of particular interest will be measurements of the extent of domain closure produced by kainate compared with data obtained for GluR2. Kainate acts as a partial agonist for GluR2, producing approximately 10° less domain closure than glutamate or AMPA. Coupled with this analysis, we plan to design non-desensitizing kainate receptors and use them to quantify the action of partial agonists, as we have done for AMPA receptors.

Until now, we have not succeeded in obtaining diffraction-quality crystals for GluR5 and thus will continue optimization of crystallization conditions. GluR5 is of special interest because recent studies show that GluR5 antagonists have anticonvulsant activity. A large number of GluR5-selective ligands that do not bind to GluR6 have been developed, including ATPA, 5-iodowillardiine, and the (S)-enantiomer of E-4-(2,2-dimethylpropylidene) glutamic acid, but the structural basis for this selectivity is not well understood. Attempts to express soluble KA1 and KA2 have not met with success, and so far sparse matrix folding screens have not yielded sufficient protein from solubilized inclusion bodies for structural work. We will examine different expression systems for these proteins.

### Structural Basis for Glutamate Receptor Desensitization

*Horning, Mayer; in collaboration with Gouaux*

Receptor desensitization is a common process that occurs in molecules as diverse as ion channels and G protein–coupled receptors.

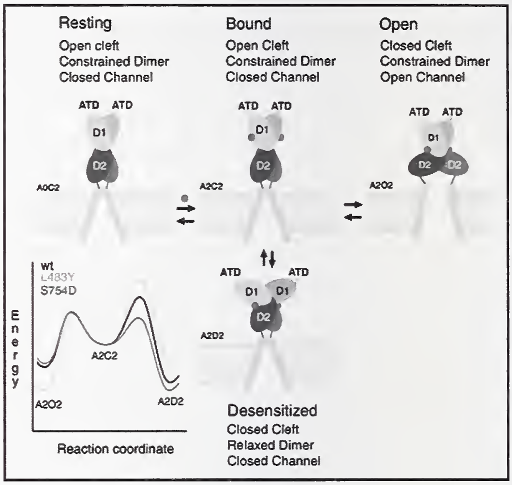

**FIGURE 15**

Mechanistic scheme for GluR desensitization based on crystal structures of the ligand binding core.

While the role(s) of desensitization may differ among different pathways, the concept that desensitization participates in shaping the amplitude, duration, and frequency of signals at synaptic circuits is gaining acceptance. Despite the fact that desensitization is a ubiquitous phenomenon in receptor signal transduction cascades, the molecular mechanisms underlying desensitization have defied analysis. By performing structural and functional studies on the AMPA-selective GluR2 receptor that localize crucial elements of the receptor involved in desensitization, we have obtained evidence for quantitative relationships between the extent of receptor desensitization and the strength of intradimer subunit interface interactions. Our goal was to test whether the interactions that are seen in the dimer interface in the crystal structures are present in the intact receptors and to determine the extent to which the strength of the dimer interface, as measured by the dimer dissociation constant ($K_d$) using analytical ultracentrifugation experiments, is correlated with the degree of receptor desensitization. To this end, we made a number of single, double, and triple mutants and examined their behaviors by using the patch clamp technique and rapid solution exchange. Our analyses revealed a remarkable correlation between the extent of equilibrium desensitization and the $K_d$ of dimer dissociation for mutations at the binding site for the allosteric modulator cyclothiazide and at the position 483 mutation from leucine to tyrosine, which blocks desensitization. Our experiments suggest a model in which the dimer interface acts as a supporting point so that the conformational strain caused by ligand binding–evoked domain closure can be transferred to the gate, which we assume is located at the extracellular surface of the membrane, thus opening the channel. In the event of desensitization, the ligand-binding cleft also closes and traps agonist, but the dimer interface is disrupted, thus releasing the domain closure energy and decoupling it from the channel gate. For the wild-type receptor, the energy barrier for desensitization is higher than for activation; therefore, the receptor activates faster than it desensitizes. The desensitized receptor is more stable than the activated receptor, however, and thus, during a prolonged incubation with agonist, the large share of the receptors becomes desensitized.

## SELECTED PUBLICATIONS

1. Hogner A, Kastrup JS, Jin R, Liljefors T, Mayer ML, Egebjerg J, Larsen IK, Gouaux E. Structural basis for AMPA receptor activation and ligand selectivity: crystal structures of five agonist complexes with the GluR2 ligand binding core. J Mol Biol. 2002;322:93.
2. Sun Y, Olson R, Horning M, Armstrong N, Mayer M, Gouaux E. Mechanism of glutamate receptor desensitization. Nature. 2002;417:245-253.

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# HIPPOCAMPAL INTERNEURONS AND THEIR ROLE IN CONTROLLING EXCITABILITY

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GABAergic inhibitory interneurons constitute a population of hippocampal cells whose high degree of anatomical and functional divergence make them suitable candidates for controlling the activity of large populations of principal neurons. They play a major role in the synchronization of neuronal activity and are involved in the generation of large-scale network oscillations. Thus, interneurons function as a clock that dictates when principal cells fire during suprathreshold excitatory drive. Interneurons receive strong excitatory glutamatergic innervation via numerous anatomically distinct afferent projections, and recent evidence has demonstrated that the molecular compositions of both classes of AMPA-preferring glutamate receptors expressed at interneuron synapses are often distinct from those found at principal cell synapses. Furthermore, single inhibitory interneurons can synthesize distinct AMPA receptors with defined subunit composition and target them to synaptic domains innervated by different afferent inputs. By using high-resolution whole-cell patch clamp recording techniques in brain slices of the hippocampus and the auditory and barrel cortex, we investigate differential mechanisms of synaptic transmission onto hippocampal inhibitory interneurons and the role of intrinsic voltage-gated channels in regulating interneuron excitability.

## Electrotonic Analysis of Strontium-Induced Asynchronous Events at Mossy Fiber–CA3 Interneuron Synapses

*Lawrence*

Network excitability in CA3 is determined in part by the capacity of mossy fibers to engage interneurons in feed-forward inhibition. We have previously used variance-mean (VM) analysis to estimate the probability of release, the number of release sites, and the quantal amplitude (q) for mossy fiber (MF) transmission onto CA3 interneurons. We have corroborated our estimate of q at MF synapses by evoking asynchronous events in the presence of 4-8 mM strontium. Rapidly rising EPSCs, presumably from proximal synapses, were evoked at low stimulus intensities. To distinguish strontium-induced asynchronous events from background spontaneous activity, we separated events with rise times similar to the synchronous component from slower-rising, presumably more distally occurring, events. Five to 20 milliseconds (ms) after the synchronous component, the frequency of these asynchronous events increased to about nine times the baseline frequency and then decayed exponentially (with a $\tau$ of the order of 125 ms) to a rate not significantly different from that seen in the absence of strontium. In contrast, the frequency of the slower events remained unchanged at all latencies. We calculated the average q by collecting fast-rising asynchronous events at latencies of between 5 and 260 ms after the synchronous component, at which time the probability of contamination by fast-rising spontaneous events was less than 20 percent. The average q was about 22 pA, consistent with that obtained independently through VM analysis of single-fiber inputs (about 27 pA). Combining this level of analysis of mossy fiber synaptic transmission with compartmental models of CA3 interneurons, we are currently estimating the attenuation in quantal amplitude due to electrotonic location at MF synapses. Such analyses will provide powerful insights into the computational abilities of interneurons within the CA3 hippocampal network.

### Differential Expression of NMDA Receptors at Mossy Fiber–Stratum Lucidum Interneuron Synapses

*Lei*

MF-pyramidal neuron synapses differ from most central synapses in that they contain few NMDA receptors (NMDAR). The ratio of NMDAR- to AMPAR-mediated EPSCs at MF-principal neuron synapses is one-third of that found at CA3-associated-commissural-principal cell synapses. Moreover, one of the hallmarks of the MF-principal cell synapse is its apparent lack of NMDAR-dependent synaptic plasticity. While the nature and function of AMPARs at MF–interneuron synapses are well described, there has been no confirmation of the existence of NMDA receptors at either synapse type. Furthermore, the potential for differential distribution and properties of NMDARs at synapses containing $Ca^{2+}$-permeable (CP) or $Ca^{2+}$-impermeable (CI) AMPARs has not been explored.

We demonstrated that the extent of philanthotoxin (PhTx) inhibition of synaptic AMPA receptors varied significantly between synapses, indicating a heterogeneous level of GluR2 expression. The data suggested that MF–interneuron synapses consist of a continuum that ranged from CP-AMPAR to CI-AMPAR–containing synapses. The amplitudes of evoked NMDAR-mediated EPSCs were negatively correlated with the degree of PhTx block of the AMPA EPSCs. The NMDAR/AMPAR ratio at CP synapses was about one-fourth of that at CI synapses. In addition, the decay time constant of the NMDAR-mediated component was markedly lower at CP than CI synapses. The sensitivity to ifenprodil, a specific blocker of NR2B-containing NMDARs, was much higher, and the open probability, probed with the irreversible open channel blocker MK-801, was smaller at CP than at CI synapses. Taken together, these data likely account for the larger amplitude and more rapid NMDAR EPSCs at CI synapses. In contrast, estimates of the single-channel conductance using nonstationary noise analysis revealed no difference in the single channel conductances of NMDARs (about 50pS) at either CI or CP synapse.

The lack of GluR2 in AMPARs of CP synapses was correlated with the presence of NR2B subunits in NMDARs and vice versa. Within many CNS regions, NR2B and GluR2 expression is developmentally regulated, with high NR2B and low GluR2 predominating early in development. To address whether the absence of GluR2 reflected developmentally immature MF–interneuron synapses, we examined four age groups spanning stages at which MF–interneuron synapses appear (Postnatal day (P) 9–10), mature (P15–16), and then fully developed (P20–21 and P30–40). At P9–10, CP synapses were the predominant synapse type observed (61.3 percent) while CI synapses predominated at P30–40 (45.6 percent). The intermediate age group of P15–16 contained the highest percentage of synapses comprised of "mixed" AMPARs, suggesting that P15–16 may represent a period of transition from CP to CI synapses. Therefore, although both CI and CP AMPAR synapses were developmentally regulated, both synapse types were observed in considerable numbers at all developmental stages, suggesting that the two synapse types correspond to functionally distinct MF-interneuron synapses. Of particular interest, the subunit composition of AMPARs and NMDARs at mossy fiber synapses is not independent. One possible explanation is that the expression of the two subunits is coregulated within the framework of a common genetic program. Alternatively, selective protein-protein interactions may constrain the assembly and subunit composition of the two receptor types in the post-synaptic density.

### Activation of Kinetically Distinct Synaptic Conductances on Inhibitory Interneurons by Electrotonically Overlapping Afferents

*Lawrence, Walker*

We have shown previously that single CA3 str. lucidum inhibitory neurons target AMPA receptors with molecular compositions distinct from those on synapses formed by specific afferent inputs. Thus, single interneurons receive input from MF of dentate gyrus granule cells onto either CP or CI AMPA receptors while recurrent collaterals of CA3 pyramidal neurons synapse exclusively onto CI AMPA receptors. While these afferent-specific populations of AMPA receptors are pharmacologically distinct, the spatial distribution of these synapses along the dendritic arbor is unknown. Traditional methods such as paired recordings have been problematic because of the exceedingly low probability of connected pairs in dual dentate granule cell–CA3 interneuron recordings; a given granule cell axon contacts only 40 to 50 inhibitory neurons in the CA3 hippocampus, separated over a considerable distance. Consequently, due to the uncertainty in electrotonic distances and distortion of somatically recorded synaptic currents attributable to dendritic filtering, it was not known whether the underlying afferent-specific synaptic conductances were associated with different time courses. Given that the time course of the synaptic conductance is a major determinant of how neurons integrate afferent input, an accurate estimate of this parameter is required to understand fully how either feed-forward or feedback excitation engages the post-synaptic inhibitory interneuron.

We circumvented the problem of dendritic filtering by adopting a somatic “voltage jump” technique that involves applying hyperpolarizing voltage jumps from the somatic recording electrode at various times after onset of an evoked synaptic current. If the receptors of interest are open when the voltage jump reaches the site of the synapse, the increase in the driving force draws additional charge through the channels. Conversely, if conductance has ended and the receptors are closed, no additional charge will be recovered. The time course of the recovered charge yields the “true” time course of the conductance occurring at the synapse. Moreover, because the time course of the change in driving force that reaches the synapse is related to the distance between soma and synapse, the voltage jump method also provides valuable information regarding the electrotonic distribution of synapses along the somatodendritic axis.

We found that two afferent-specific populations of AMPA receptors were associated with different excitatory synaptic conductance time courses but overlapped in electrotonic location. Mossy fiber synaptic conductances were on average nearly twice as rapid as recurrent collateral synapses (1.5 versus 2.7 msec). Interestingly, electrotonic information revealed that mossy fiber inputs tended to be clustered at more proximal locations to the somata while recurrent collateral inputs were diffusely distributed across the entire dendritic tree. Thus, afferent-specific conductance time courses allow single interneurons to integrate feed-forward and feedback information differentially without the need to segregate distinct AMPA receptor subunits to different electrotonic domains.

### GABA$_B$ Receptor–Mediated Regulation of Excitatory and Inhibitory Synaptic Transmission

*Lei*

CA3 stratum radiatum inhibitory interneurons are a heterogeneous population that receive both excitatory synaptic innervation from the collateral recurrent fibers of CA3 pyramidal neurons and inhibitory synaptic transmission from other GABAergic interneurons. GABA$_B$ receptor–mediated post-synaptic IPSP(C)s have been described in interneurons throughout the hippocampus, including CA1 stratum pyramidale, CA1 lacunosum-moleculare, and dentate-hilus border. However, to our surprise, GABA$_B$ receptor–mediated modulation of presynaptic glutamate and GABA release at hippocampal interneuron synapses has not been elucidated. Since activation of presynaptic GABA$_B$ receptors may be an important step in modulation of short- (and long-) term synaptic plasticity, we investigated whether GABA$_B$ receptors were present on either excitatory or inhibitory synaptic terminals onto CA3 interneurons and asked whether their activation altered the dynamics of transmission at either of these synapses.

We demonstrated that activation of $GABA_B$ receptors by baclofen inhibited the amplitudes of both evoked AMPA receptor–mediated EPSCs and $GABA_A$ receptor–mediated IPSCs with $IC_{50}$s of 8.5 and 1.7 μM, respectively. Baclofen enhanced the paired-pulse ratio and coefficient of variation of evoked EPSCs and IPSCs but reduced the frequencies of spontaneous and KCl-evoked miniature EPSCs and IPSCs, consistent with a presynaptic mechanism. Baclofen blocked the frequency-dependent depression of both EPSCs and IPSCs but was ineffective at blocking frequency-dependent facilitation of EPSCs. Whereas N- and P/Q-types of $Ca^{2+}$ channels contributed equally to $GABA_B$ receptor–mediated inhibition of EPSCs, more P/Q-type $Ca^{2+}$ channels were involved in $GABA_B$ receptor–mediated inhibition of IPSCs. Taken together, our data demonstrate that presynaptic $GABA_B$ receptors are expressed on the terminals of both excitatory and inhibitory synapses onto CA3 interneurons and that their activation modulates essential components of the release process to modulate transmission at these two synapse types.

### The Role of the Kainate Receptor Subunits GluR5 and GluR6 in Hippocampal Gamma Oscillations

*Fisahn; in collaboration with Heinneman, Buhl*

Activation of kainate receptors (but not AMPA receptors) induces gamma oscillations in the hippocampus *in vitro*, oscillations that depend on intact inhibitory GABAergic neurotransmission but not on excitatory transmission via NMDA receptors, metabotropic glutamate receptors, or cholinergic receptors. We investigated the connection between gamma oscillations and the kainate receptor subtypes GluR5 and GluR6. Using extracellular field recordings, we demonstrated that gamma oscillations of comparable power were induced by much lower concentrations of kainate in GluR5-/- (100nM) CA3 hippocampus than in wild type (600nM). Increasing concentrations of kainate (>150nM) degraded the gamma oscillation and precipitated electrographic seizure activity in GluR5-/- hippocampus but not in wild type. In contrast, kainate failed to induce gamma oscillations or seizures at concentrations up to 600nM in GluR6-/- hippocampus. Given that gamma oscillations were readily evoked by other oscillogenic drugs such as muscarine (20μM), this lack of effect of kainate was not explained by altered neuronal circuitry in GluR6-/- mice. We concluded that activation of the GluR6 kainate receptor subtype is necessary for the induction of gamma oscillations. Next, using whole cell recordings, we demonstrated that kainate increased sIPSC amplitude by about 50 percent in wild type and GluR5-/- but was without effect on sIPSCs in GluR6-/-. Furthermore, kainate activated an inward current and depolarized pyramidal neurons in both wild type and GluR5-/- but not in GluR6-/-. These effects of kainate on the hippocampal network are consistent with the expression patterns of GluR5 and GluR6, which are primarily localized on inhibitory interneuron presynaptic terminals and somata and dendrites of interneurons and pyramidal cells, respectively. Consistent with a presynaptic expression pattern of GluR5, the paired-pulse ratio of evoked IPSCs was increased, suggesting that GluR5 may act to depolarize presynaptic inhibitory terminals tonically, providing an elevated basal state of inhibition within the hippocampal network. Similarly, the increased sensitivity to kainate-induced oscillations in the GluR5-/- likely results from a reduction of the inhibitory control over the CA3 network, leading to an enhanced excitability of pyramidal neurons and a reduced ability of the interneuron network to synchronize itself at gamma frequencies.

## SELECTED PUBLICATIONS

1. Atzori M, Lei S, Evans DI, Kanold P, Phillips-Tansey E, McIntyre O, McBain CJ. Differential synaptic processing separates stationary from transient inputs to the auditory cortex. Nat Neurosci. 2001;4:1230-1237.

2. Chittajallu R, Chen Y, Wang H, Yuan X, Ghiani CA, Heckman T, McBain CJ, Gallo V. Regulation of Kv1 subunit expression in oligodendrocyte progenitor cells and their role in G1/S phase progression of the cell cycle. Proc Natl Acad Sci USA. 2002;99:2350-2355.
3. Fisahn A, Yamada M, Duttaroy A, Gan JW, Deng CX, McBain CJ, Wess J. Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. Neuron. 2002;33:615-624.
4. Lei S, McBain CJ. Distinct NMDA receptors provide differential modes of transmission at mossy fiber-interneuron synapses. Neuron. 2002;33:921-933.
5. McBain CJ, Fisahn A. Interneurons unbound. Nat Rev Neurosci. 2001;2:11-23.
6. McBain CJ, Rudy B. Kv3 Channels: Voltage-gated K+ channels designed for high-frequency repetitive firing. Trends Neurosci. 2001;24:517-526.
7. Phillips-Tansey E, McBain CJ. 2002 Developmental expression of potassium channel subunit Kv3.2 within subpopulations of hippocampal inhibitory interneuron. Hippocampus. 2002;12:337-348.
8. Walker HC, Lawrence JJ, McBain CJ. Activation of kinetically distinct synaptic conductances on inhibitory interneurons by electrotonically overlapping afferents. Neuron. 2002;35:161–171.

## COLLABORATORS

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# POTASSIUM CHANNELS AND DENDRITIC FUNCTION IN HIPPOCAMPAL PYRAMIDAL NEURONS

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A nonuniform density of subthreshold, rapidly inactivating potassium channels regulates signal propagation in the dendrites of CA1 pyramidal neurons of the hippocampus. This nonuniform density (with higher expression in the dendrites than in the soma) means that the electrical properties of the dendrites are markedly different from those of the soma. Incoming synaptic signals are shaped by the activity of these channels, and action potentials, once initiated in the axon, progressively decrease in amplitude as they propagate back into the dendrites. By combining patch clamp recording in brain slices of hippocampus with molecular biology techniques, the Molecular Neurophysiology and Biophysics Unit investigates the electrophysiological properties and molecular nature of the voltage-gated channels expressed in CA1 dendrite, how their expression is regulated, and their role in synaptic integration.

## SELECTED PUBLICATIONS

1. Hoffman DA, Sprengel R, Sakmann B. Molecular dissection of hippocampal theta-burst pairing potentiation. Proc Natl Acad Sci USA. 2002;99:7740-7745.
2. Johnston D, Hoffman DA, Magee JC, Poolos NP, Watanabe S, Colbert CM, Migliore M. Dendritic potassium channels in hippocampal pyramidal neurons. J Physiol. 2000;525 Pt 1:75-81.
3. Watanabe S, Hoffman DA, Migliore M, Johnston D. Dendritic $K^+$ channels contribute to spike-timing dependent long-term potentiation in hippocampal pyramidal neurons. Proc Natl Acad Sci USA. 2002;99:8366-8371.

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# REGULATION OF NEURONAL GENE EXPRESSION BY ACTION POTENTIALS

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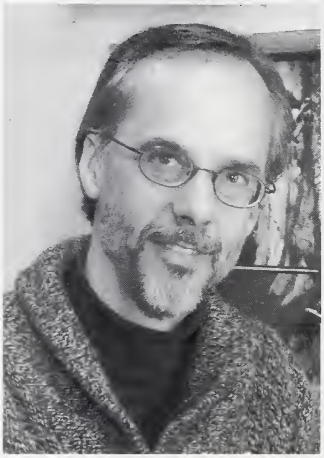

Our research is concerned with understanding the molecular mechanisms by which functional activity in the brain regulates development of the nervous system during late stages of fetal development and early postnatal life. The main objectives of our research program are to understand how the expression of genes controlling the developing structure and function of the nervous system are regulated by patterned neural impulse activity and to determine the functional consequences of neural impulse activity on major developmental processes, including cell proliferation, survival, differentiation, growth cone motility, axon bundling (fasciculation), neurite outgrowth, synaptogenesis and synapse remodeling, myelination, interactions with glia, and the mechanisms of learning and memory in postnatal animals.

## Action Potential Regulation of Glial Development

*Stevens, Fields; in collaboration with Gallo, Weinberg*

While the importance of neural impulse activity in regulating development of neurons is widely appreciated, the same does not hold for development of non-neuronal cells (glia). Indeed, in view of the intimate structural and functional association between neurons and glia and the powerful influence of glia on nervous system development, it is surprising that the importance of glial cells has not captured more attention. Our recent work showed that Schwann cells [myelin-forming glia of the peripheral nervous system (PNS)] can detect action potentials in premyelinated axons. In addition, it identified the neuron-glial signaling molecule mediating this communication (extracellular ATP); showed that ATP was released by axons firing action potentials and identified the membrane receptors on Schwann cells that were involved (P2Y receptors); identified the intracellular signaling pathways in Schwann cells activated by action potentials in axons; and showed that several genes with important developmental functions in Schwann cells were regulated by neural impulses communicated via activity-dependent release of ATP from axons. We found that several developmental processes are regulated by neural impulse activity acting through the release of ATP, including Schwann cell proliferation, differentiation, and myelination.

Neural impulse conduction depends on the insulating layers of myelin membrane wrapped around axons by glial cells. Interestingly, our work indicates that impulse activity inhibits myelination in the PNS, but research from other laboratories suggests that, in the central nervous system (CNS), impulse activity stimulates myelination through an unknown mechanism.

We investigated this controversy by taking advantage of a preparation of DRG neurons that can be stimulated in cell cultures fitted with platinum electrodes. The central axons of DRG neurons are myelinated by oligodendrocytes, and their peripheral axons are myelinated by Schwann cells.

It is not known if oligodendrocytes can respond to action potentials in premyelinated axons or whether ATP or another activity-dependent signaling molecule can regulate oligodendrocyte development. By using confocal calcium imaging, our research reveals that oligodendrocyte progenitor cells (OPCs) can detect impulse activity in premyelinated axons. We identified the involved neuron-glial signaling molecule and found that adenosine, a breakdown product of ATP, was of primary importance. Using PCR, we detected all four types of adenosine receptor in purified cultures of OPCs and in OPCs freshly dissociated from transgenic mice carrying a GFP gene driven by the CNP promoter, which is expressed only in cells of oligodendroglial lineage in the CNS.

We identified several functional effects in OPCs that were regulated by neural impulse activity acting through extracellular adenosine, including control of OPC proliferation, differentiation, and myelination. Impulse activity inhibited cell proliferation for myelinating glia in both the PNS and CNS, but the effects of impulse activity on differentiation and myelination were opposite in Schwann cells and OPCs. In contrast to Schwann cells, in which our experiments showed that electrical activity arrests differentiation, we found that action potentials acting through adenosine receptors promoted differentiation of OPCs to the mature stage, evidence that was obtained by using both specific antigenic markers of differentiation (O4, O1, and MBP) and digital morphometry analyzed by multivariate cluster analysis and then replicated the effects in brain slice preparations of rat cerebellum. When cultures of DRG neurons were myelinated by oligodendrocytes, we found that the addition of adenosine significantly increased myelination. Electrical stimulation of axons also increased myelination, which adenosine receptor antagonists blocked.

Our findings that different extracellular signaling molecules mediate axon communication with OPCs in the CNS and Schwann cells in the PNS help to resolve a long-standing controversy over the opposite effects of impulse activity on myelination in the brain and PNS. Our research identifies adenosine as a potent axon-derived differentiation factor for OPCs that operates in an activity-dependent manner and promotes myelination. Together with our research detecting several types of purinergic receptors in OPCs, the findings open new avenues of research into oligodendrocyte development with potential for new therapeutic approaches to treating demyelinating disease.

### Gene Expression in Conversion of Short-Term into Long-Term Memory

*Dudek,[b] Fields*

It is widely appreciated that there are two types of memory: short-term and long-term. It has been well known for decades that gene expression is necessary for converting short-term into long-term memory, but it is not known how signals reach the nucleus to initiate the process. Current theory holds that, in response to firing a synapse in the appropriate manner to generate a short-term memory, a signaling molecule is generated from the submembrane region of the synapse and then travels up the dendritic tree to the cell body. After entering the nucleus, the signaling molecule is presumed to initiate gene transcription by activating the transcription factor CREB. The gene product is synthesized and distributed throughout the dendritic tree, but the protein can recognize synapses that have been temporarily strengthened, rendering the strengthening permanent.

Based on our studies of action potential-dependent intracellular signaling and CREB activation in DRG neurons, we proposed an alternative hypothesis. It posited that, rather than generating a specialized synapse-to-nucleus signaling molecule, *somatic* action potentials could be responsible for activating gene transcription to convert short-term into long-term memory via the same intracellular signaling pathway that we studied previously in DRG neurons. We tested the hypothesis by causing neurons to fire somatic action potentials in the absence of all excitatory synaptic input in brain slice preparations. More specifically, we were able to backfire axons from CA1 hippocampal neurons in slice preparations in the presence of glutamate neurotransmitter blockers. Following stimulation, we analyzed the slices by immunocyochemistry for activation of CREB and other signaling enzymes involved in long-term potentiation (LTP) of synapses and for expression of genes closely associated with LTP induction. The results showed that backfiring the axons by antidromic stimulation was sufficient to activate CREB and the signaling enzyme MAPK in CA1 neurons in hippocampal slice in the absence of all excitatory synaptic stimulation.

Given that somatic action potential firing in the absence of all excitatory synaptic activity activated the appropriate intracellular signaling pathways, transcription factors, and genes, we tested whether antidromic stimulation was sufficient to convert e-LTP to l-LTP, a cellular model of conversion of short-term memory into long-term memory. When slices were stimulated antidromically, synaptic stimulation that normally results in a transient strengthening of the synapse (e-LTP) resulted instead in long-lasting strengthening of the synapse (l-LTP). The results support our hypothesis and eliminate the necessity of a synapse-to-nucleus signalling molecule for l-LTP. In addition, the results are consistent with the original theory of Hebb that states that the firing of a neuron determines whether or not it forms a stronger synaptic connection with another neuron.

### Role of CaMKII in Decoding Action Potential Frequency

*Eshete,[c] Fields*

A fundamental problem in cellular neuroscience is in understanding how action potential firing patterns are transmitted and integrated within intracellular signaling cascades to regulate neuronal responses to appropriate patterns of action potential firing. It has been theorized for more than a decade that the unique properties of a calcium-dependent kinase (CaMKII) that involves autophosphorylation at Thr-286, which renders the enzyme active in the absence of calcium, could allow the kinase to function as a "molecular switch" or spike frequency–decoding molecule. The theory was based on mathematical modeling and *in vitro* simulations but, before our work, had not been tested experimentally. The properties of the kinase and the experiments showing interference with LTP during blockage of CaMKII function formed the basis for the widely accepted view that the molecule plays a pivotal role in hippocampal LTP and LTD. In work published this year, we presented evidence in support of the hypothesis that CaMKII can decode action potential firing patterns in neurons and showed that the frequency of intracellular calcium transients can be more important than the magnitude of change in calcium concentration evoked by action potentials.

We took advantage of our preparation of mouse DRG neurons, which can be stimulated to fire action potentials over a wide range of frequencies in a controlled manner in cell culture. Biochemical methods, including *in vitro* kinase assays, Western blot, and immunocytochemistry, together with live cell confocal calcium imaging allowed us to test the hypothesis by applying controlled patterns of action potentials. Our work showed that the enzyme could decode action potential firing patterns, but the frequency response was sharply tuned to low frequencies of action potentials (less than 1 Hz). Moreover, we found that the enzyme was in a highly autophosphorylated state before stimulation, an observation previously made by others but mistakenly attributed to spontaneous synaptic activity. The high level of autophosphorylation, together with the long duration of calcium transients induced by action potentials (about 300 ms), severely limited the frequency response of the enzyme. Surprisingly, we also found that the enzyme was highly sensitive to the concentration of extracellular calcium. If CaMKII is to decode frequencies of calcium transients induced by patterns of stimulation required to induce LTP (about 100 Hz), the results of our work suggest that the enzyme must be segregated within a specialized subcellular compartment where the calcium dynamics are of shorter duration, that the enzyme is not highly autophosphorylated in the basal state, and that phosphatases are active in reducing phosphorylation of CaMKII at Thr-286.

Dendritic spines or subcellular microdomains might provide such an environment.

### Activity-Dependent Regulation of Gene Expression

*Lee, Tendi, Field; in collaboration with Becker*

Using cDNA arrays for gene expression profiling, we have investigated how gene expression is regulated by patterned action potential and synaptic activity in cell culture and the hippocampal slice. Our work indicates that expression of large sets of genes depends on the pattern of neural impulse firing and is revealing signalling pathways and gene regulatory networks that respond selectively to appropriate temporal patterns of action potential and synaptic firing. Similarly, gene expression profiling has provided novel insights into signalling pathways in Schwann cells responding to extracellular ATP and into the molecular basis for neurofibromatoma type 1 formation in human Schwann cells with mutations in the NF-1 gene.

## SELECTED PUBLICATIONS

1. Dudek SM, Fields RD. Mitogen-activated protein kinase/extracellular signal-regulated kinase activation in somatodendritic compartments: roles of action potentials, frequency and mode of calcium entry. J Neurosci. 2001;21,RC122:1-5.
2. Dudek SM, Fields RD. Somatic action potentials are sufficient for late-phase LTP-related cell signaling. Proc Natl Acad Sci USA. 2002;99:3962-3967.
3. Eshete F, Fields RD. Spike frequency decoding by autonomous activation of CaMKII in DRG neurons. J Neurosci. 2001;21:6694-6705.
4. Fields RD, Stevens B. ATP in signaling between neurons and glia. Trends Neurosci. 2000;23:625-633.
5. Fields RD, Stevens B. New views of neuron-glia communication. Science. 2002;298:556-562.
6. Stevens B, Fields RD. Action potentials regulate Schwann cell proliferation and development. Science. 2000;287:2267-2271.
7. Stevens B, Fields RD. Regulation of the cell cycle in normal and pathological glia. Neuroscientist. 2002;8:93-97.
8. Stevens B, Porta S, Haak LL, Gallo V, Fields RD. Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. Neuron. 2002;36:855-868.

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# NEUROTROPHIC REGULATION OF SYNAPSE DEVELOPMENT AND PLASTICITY

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We study the molecules that regulate the development and function of synapses, with particular emphasis on the role of neurotrophic factors. Traditionally, neurotrophic factors are defined as secretory proteins that regulate neuronal survival and differentiation. Recent studies have established a new concept, that is, neurotrophic factors also play important roles in synapse transmission and plasticity in both the developing and adult nervous systems. This laboratory was among the first to study the synaptic functions of neurotrophic factors. Two types of regulation have been discovered: *acute* modulation of synaptic transmission and plasticity and *long-term* alteration of the structure and function of synapses. Continuing studies in this emerging field will help illuminate how synapses develop and function in the brain and may have general implications for treating neurological disorders in both children and adults.

## Pre- and Post-Synaptic Effect of GDNF and Neurturin on the Development of Neuromuscular Synapses

*Wang, Yang, Je, Lu*

Glial cell line–derived neurotrophic factor (GDNF) is known for its potent effect on neuronal survival, but its role in the development and function of synapses is not well studied. Using *Xenopus* nerve–muscle co-cultures, we showed that GDNF and its family member neurturin (NRTN) facilitate the development of the neuromuscular junction (NMJ). Long-term application of GDNF significantly increased the total length of neurites in the motoneurons. GDNF also caused an increase in the number and size of synaptic vesicle clustering, as demonstrated by synaptobrevin-GFP fluorescent imaging and FM dye staining.

Electrophysiological experiments revealed two effects of GDNF on synaptic transmission at NMJ. First, GDNF markedly increased the frequency of spontaneous transmission and decreased the variability of evoked transmission, suggesting an enhancement of transmitter secretion. Second, GDNF elicited a small increase in the quantal size without affecting the average rise and decay times of synaptic currents. Imaging analysis showed that the size of acetylcholine receptor (AChR) clusters at synapses increased in muscle cells over-expressing GDNF. Neurturin had effects very similar to those of GDNF. The results suggest that GDNF and NRTN are new neuromodulators that regulate the development of the neuromuscular synapse through both pre- and post-synaptic mechanisms.

## Regulation of AChR Clustering by Dishevelled Interacting with MuSK and PAK1

*Lou, Wang, Zhou, Lu, Mei*

An important aspect of synapse development is the clustering of neurotransmitter receptors in the post-synaptic membrane. The best-known molecule that controls AChR clustering at the NMJ is Agrin. Although MuSK is required for Agrin-induced AChR clustering, the underlying molecular mechanisms remain unclear. We showed that, in muscle cells, MuSK interacts with Dishevelled (Dvl), a signalling molecule important for planar cell polarity. Disruption of the MuSK-Dvl interaction inhibits Agrin-induced and neuron-induced AChR clustering. Expression of dominant negative Dvl1 in post-synaptic muscle cells reduces the amplitude of spontaneous synaptic currents at the NMJ. Moreover, Dvl1 interacts with a downstream kinase PAK1. Agrin activates PAK, and the activation requires Dvl. Inhibition of PAK1 activity attenuates AChR clustering. The results demonstrate important roles of Dvl and PAK in Agrin/MuSK-induced AChR clustering and reveal a novel function of Dvl in synapse development.

### Synaptic Vesicle Depletion in Mice Lacking α-Synuclein

*Shimazu, Lu; in collaboration with Cabin, Nussbaum*

While mutation of α-synuclein, a protein associated with pre-synaptic vesicles, is implicated in the etiology and pathogenesis of Parkinson's disease, the biological function of the normal protein is unknown. Mice lacking α-synuclein have been generated. Electron microscopic examination of hippocampal synapses revealed a selective deficiency of undocked vesicles without affecting docked vesicles. Field recording of CA1 synapses in hippocampal slices from the mutant mice demonstrated normal basal synaptic transmission, paired pulse facilitation, and response to a brief train of high-frequency stimulation (100 Hertz, 40 pulses) that exhausts only docked vesicles. In contrast, the α-synuclein knockout mice exhibited significant impairments in synaptic response to a prolonged train of repetitive stimulation (12.5 Hertz, 300 pulses) capable of depleting docked as well as reserve pool vesicles. Moreover, the replenishment of the docked vesicles by reserve pool vesicles after depletion was slower in the mutant synapses. Thus, α-synuclein may be required for the genesis and/or maintenance of a subset of presynaptic vesicles, those in the "reserve" or "resting" pools. These results reveal, for the first time, the normal function of endogenous α-synuclein in regulating synaptic vesicle mobilization at nerve terminals (Cabin *et al.*, J Neurosci. 2002).

## SELECTED PUBLICATIONS

1. Cabin DE, Shimazu K, Murphy D, Cole N, Gottschalk WA, McIlwain K, Orrison B, Chen A, Garret L, Ellis C, Paylor R, Lu B, Nussbaum RL. Synaptic vesicle depletion in mice lacking alpha-synuclein. J Neurosci. 2002;22:8797-8807.
2. Luo ZG, Wang Q, Zhou JZ, Wang JB, Liu MY, He X, Wynshaw-Boris A, Xiong WC, Lu B, Mei L. Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron. 2002;35:489-505.
3. Shen L, Pichel JG, Mayeli T, Sariola H, Lu B, Westphal H. GDNF haploinsufficiency causes sporadic hirschspung-like intestinal obstruction and early onset lethality in mice. Am J Hum Genet. 2001;70:435-447.
4. Tartaglia N, Du J, Tyler WJ, Neale E, Pozzo-Miller L, Lu B. Protein synthesis dependent and independent regulation of hippocampal synapses by brain-derived neurotrophic factor. J Biol Chem. 2001;276:37585-37593.
5. Wang C, Yang F, He X, Je HS, Zhou JZ, Eckermann K, Kawamura D, Feng L, Shen L, Lu B. Regulation of neuromuscular synapse development by glial cell line-derived neurotrophic factor and neurturin. J Biol Chem. 2002;277:10614-10625.
6. Yang F, Feng L, Fang Z, Johnson SW, Du J, Shen L, Wu CP, Lu B. GDNF acutely modulates neuronal excitability and A-type potassium channels in midbrain dopaminergic neurons. Nat Neurosci. 2001;4:1071-1078.

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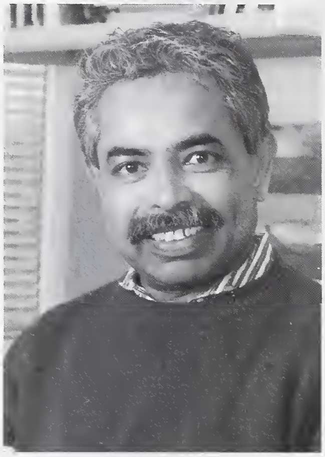
# CALCIUM-BASED EXCITABILITY IN GLIAL CELLS



**James T. Russell, D.V.M.,** *Head, Section on Cell Biology and Signal Transduction*
S. Leigh Verbois, Ph.D., *Postdoctoral Fellow*
Susanna Weerth, Ph.D., *Postdoctoral Fellow*
Lynne A. Holtzclaw, *Senior Research Assistant*

We are interested in studying the cellular mechanisms of calcium signalling by glial cells in the nervous system. Glial cells and neurons are in intimate communication with each other during central nervous system (CNS) development and normal brain function. Glial cells monitor and respond to neural activity by conditioning the extracellular milieu, signalling within glial cell networks, and sending signals back to neurons. Such signalling takes the form of propagated $Ca^{2+}$ waves that spread over long distances in response to synaptic activity. One of our objectives is to understand the processes that support temporal and spatial characteristics of $Ca^{2+}$ signals within and between cells. Another objective is to understand the precise nature of glial cell signals in response to neuronal activity and the consequence of such signals to CNS function.

## $Ca^{2+}$-Signalling Microdomains

*Yagodin, Sheppard, Simpson*

Work on astrocytes and oligodendrocyte progenitors (OP cells) in culture revealed that wave propagation is saltatory. Such propagation is achieved by assembling a number of highly specialized $Ca^{2+}$-signalling microdomains that function to produce a discretely localized spurt of cytoplasmic $Ca^{2+}$ increase.

Such focal signals regulate locally discrete cellular processes and support regenerative propagation of calcium waves through the entire cell and its processes. $Ca^{2+}$-signalling microdomains are found five to seven micrometers apart along cellular processes and are characterized by high-density patches of endoplasmic reticulum (ER) proteins such as the inositol 1,4,5-trisphosphate receptors ($IP_3Rs$), sarco-endoplasmic reticulum calcium pumps, calreticulin, and at least one mitochondrion in close association. The specialization of $Ca^{2+}$ signalling microdomains allows for enhanced $Ca^{2+}$ release at the sites. In addition to supporting long-distance wave propagation, the specialized sites provide for locally discrete $Ca^{2+}$ signals that last only for brief periods (Fig. 16).

## Local $Ca^{2+}$ Release, $Ca^{2+}$ Sparks

*Haak*

Using confocal microscopy, we recently

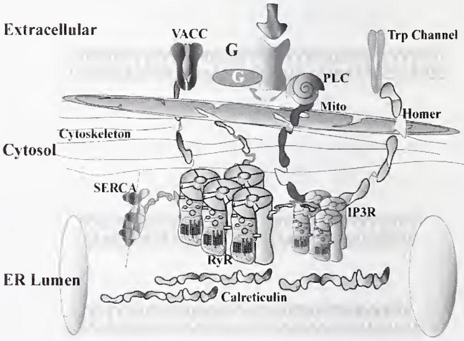


**FIGURE 16**

$Ca^{2+}$ signaling microdomains

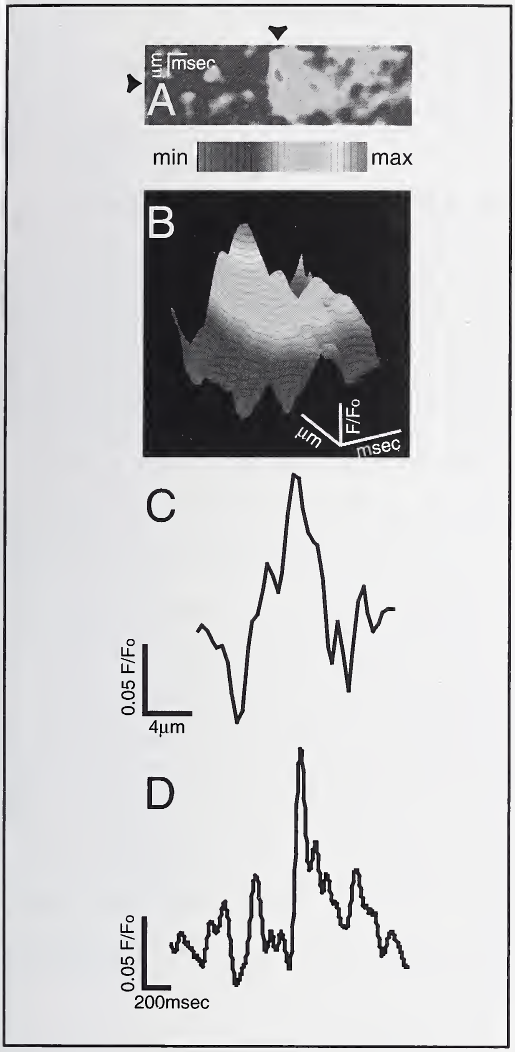


**FIGURE 17**

Ca$^{2+}$ sparks.

completed a functional characterization of the signalling rafts in OP cell processes. Our aim was to measure the kinetics of elementary Ca$^{2+}$ release events, Ca$^{2+}$ sparks, and Ca$^{2+}$ puffs (Fig. 17), which are the smallest units of local Ca$^{2+}$ release and presumed to emanate from clusters of IP3Rs on the ER. We readily recorded both spontaneous and agonist-evoked Ca$^{2+}$ sparks and puffs in OP processes. Though occurring infrequently (nine events in 51 trials), spontaneous local Ca$^{2+}$ release events were unequivocally resolved, and the amplitude of the events ranged between those of puffs and sparks. We elicited Ca$^{2+}$ sparks and puffs in OP cell processes by using a caffeine analog 3,7-dimethyl propargylxanthine (DMPX) or methacholine, respectively. Stimulation often elicited repetitive DMPX-evoked spark events, and the center of mass of the events remained unchanged, suggesting that the same RyR cluster is gated open. The abolition of DMPX-evoked elementary Ca$^{2+}$ release events by pretreating cells with ryanodine suggested that Ca$^{2+}$ release occurred through ryanodine receptors (RyRs). OP cells in culture and *in situ* express the type 3 isoform of RyRs, as shown by Western blot and immunocytochemical analyses. We found that Ca$^{2+}$ release evoked by InsP3-linked agonists elicited Ca$^{2+}$ release events through RyR channels, indicating cross-talk between the two channel types.

InsP3-stimulated Ca$^{2+}$ puffs were also readily recorded in OP cell processes. The stimulation of muscarinic m1 receptors (64 events in 34 cells) or purinergic (P2Y) receptors (12 events in seven cells) elicited such events. Depending on the stimulus intensity, sparks, macrosparks, and propagating Ca$^{2+}$ waves occurred in a hierarchical fashion and at the same cellular sites. Given that the size of the local release event is determined by the number of ion channels in the cluster and the flux through individual channels, it is not clear whether the smallest sparks represent Ca$^{2+}$ flux through individual ion channels or through a cluster of channels. The morphologies of single release events elicited by DMPX (sparks) and MeCh (puffs) differed significantly. Furthermore, specific blockers of IP$_3$Rs (Xestospongin-C, 2-APB) inhibited sparks evoked by MeCh.

Two different observations suggested that IP$_3$Rs and RyRs may modulate each other and thereby influence the local Ca$^2$ signal. Blockade of RyRs with ryanodine increased the amplitude and width of IP$_3$R-mediated puffs, pointing to a larger flux through the channels or activation of a greater number of channels. Conversely, after exposure of cells to MeCh, potentiated RyRs and DMPX-induced events increased in size. The mechanism of such modulation is currently unknown. It is likely that the two channel types may be expressed in patches, and their proximity to each other and sensitivity to ambient $Ca^{2+}$ concentration may be modulated by the activity of the other.

### $Ca^{2+}$-Signalling Micro-Domains *in Situ*

*Holtzclaw, Anant Kumar, Pandhit*

While the importance of $InsP_3$-linked signalling mechanism in glial cells, particularly in astrocytes, has been recognized for some time, the type of $IP_3Rs$ expressed by astrocytes *in situ* was not known. We undertook a study to identify and characterize the intra-cellular distribution of the $IP_3R$ isoform in astrocytes in adult rat brain. Three important observations resulted from this effort: astrocytes in adult rat brain express only $IP_3R2$; the receptor distribution is punctate and appears in patches in the cell body and the fine processes; and patchy expression of $IP_3R2s$ is observed within astrocytic processes ensheathing synaptic boutons in the neuropil. In the brain areas that we examined, astrocytes expressed only the type 2 isoform of $IP_3Rs$, suggesting that $InsP_3$-linked signalling in astrocytes *in situ* occurs predominantly, if not exclusively, through $IP_3R2$ ion channels.

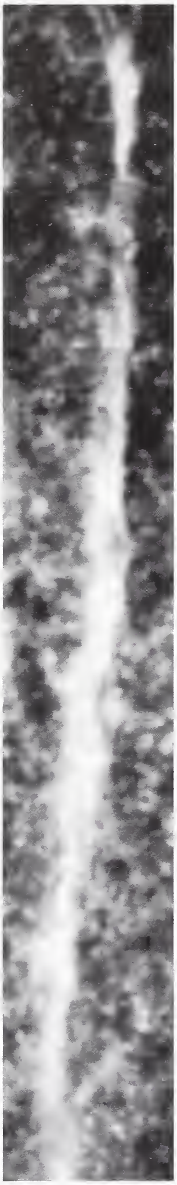


**FIGURE 18**
$IP_3R2$ in a Bergmann glial cell process

The terminal processes of astrocytes were covered with fine hair-like processes that appeared to shroud the synaptic neuropil in a cloud. Bright punctate $IP_3R2$ staining extended into these fine branches, reminiscent of astrocytic processes ensheathing individual synapses, although the puncta were not resolved by the light microscopy used in the study. We examined the staining in detail in dual indirect immunohistochemical experiments by using anti-synaptophysin to mark presynaptic terminals, anti–S-100b to mark astrocytic processes, and anti–PSD-95 to mark postsynaptic densities. We found brightly stained punctate $IP_3R2$-enriched, S-100b–containing astrocytic processes in close association with synaptophysin-containing presynaptic terminals and PSD-95–containing postsynaptic densities. Staining for synaptophysin or PSD-95 never co-localized with $IP_3R2$ staining. $IP_3R2$ staining appeared to interdigitate with PSD-95–containing profiles and synaptophysin-containing profiles.

The punctate distribution of $IP_3Rs$ in astrocytic processes in rat brain showed that $Ca^{2+}$ wave propagation *in situ* may be supported by the receptor patches. The receptor complexes function as signalling microdomains that contain other proteins and organelles involved in $Ca^{2+}$ signalling, which are scaffolded together as a signalling microdomain. The organization of astrocytic signalling machinery in proximity to neuronal elements is likely to represent glial endowment of synapses in the neuropil and to form the basis for $Ca^{2+}$-based intra- and intercellular signalling in astrocytic networks.

### Molecular Characterization of Signalling Rafts

*Verbois, Weerth, Holtzclaw*

Our current focus is on investigating the molecular organization of $Ca^{2+}$-signalling microdomains and describing their functional regulation in detail. The overall aim of our investigation is to test the hypothesis that, in the specialized microdomains of $Ca^{2+}$ release, a number of macromolecular protein complexes are physically brought together at times in different membrane systems and that they interact to generate large spatially restricted $Ca^{2+}$ signals. We have focused on glial cell processes, particularly cells of the oligodendrocyte lineage and astrocytes. We propose to undertake a molecular characterization of these specialized $Ca^{2+}$ release sites to determine the interacting protein assemblies that orchestrate this process. In addition, we propose to probe the functional significance of unique proteins found in the macromolecular assembly. We will investigate if the microdomains are preferentially localized to predestined cellular sites of contact to support optimal signalling between neurons and glial cells and between glial cells.

The goal is to isolate intact oligomeric protein assemblies that make up specialized microdomains of $Ca^{2+}$ signalling from glial cell membranes isolated from mouse brains. The enriched glial cell membrane preparations will be used in an affinity separation strategy to obtain macromolecular assemblies. We will use modern proteomic technologies for molecular identification of component protein machines that make up the specialized $Ca^{2+}$ release microdomains. The technology for proteomics involves separation of protein components in the macromolecular assembly followed by modern molecular identification using spectrometry. We will separate proteins by 1D and 2D PAGE and identify spots by staining before excising them for digestion with trypsin. Peptide pools in the tryptic digests will be identified with mass spectrometry by using MALDI-TOF. We will compare peptide mass data with protein databases for positive hits. Tandem LC-MS/MS is another technique that will allow for peptide mass determination and database comparison. We will select interesting novel proteins present in the microdomains to investigate cellular localization and functional importance.

## SELECTED PUBLICATIONS

1. Haak LL, Grimaldi M, Smaili SS, Russell JT. Mitochondria regulate $Ca^{2+}$ wave initiation and inositol trisphosphate signal transduction in oligodendrocyte progenitors. J Neurochem. 2002;80:405-415.
2. Holtzclaw LA, Pandhit S, Bare DJ, Mignery GA, Russell JT. Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. Glia. 2002;39:69-84.
3. Wang CY, Yang F, He X, Chow A, Du J, Russell JT, Lu B. $Ca^{2+}$ binding protein frequenin mediates GDNF-induced potentiation of $Ca^{2+}$ channels and transmitter release. Neuron. 2001;32:99-112.

# OLFACTORY CODING BY ENSEMBLES OF NEURONS

**Mark Stopfer, Ph.D.,** *Head, Unit on Sensory Coding and Neural Ensembles**

All animals need to know what is going on in the world around them; thus, brain mechanisms have evolved to gather and organize sensory information, thereby building transient and sometimes enduring internal representations of the animal's surroundings. Using relatively simple animal models and focusing primarily on olfaction, we combine electrophysiological, behavioral, and other techniques to examine how neural systems process information. Our research program addresses several general areas of inquiry: the mechanisms that underlie information coding and decoding, including the transient oscillatory synchronization of ensembles of neurons; the mechanisms that underlie the phenomenon of stimulus invariance, in which stimuli are perceived as the same, or of the same class, despite actual differences in orientation, intensity, and so forth; the manner in which sensory stimuli make their way into short- and long-term memories; and the manner in which multimodal stimuli are integrated into unified perceptions.

## SELECTED PUBLICATIONS

1. Bazhenov M, Stopfer M, Rabinovich M, Abarbanel HD, Sejnowski TJ, Laurent G. Model of cellular and network mechanisms for odor-evoked temporal patterning in the locust antennal lobe. Neuron. 2001;30:569-581.
2. Bazhenov M, Stopfer M, Rabinovich M, Huerta R, Abarbanel HD, Sejnowski TJ, Laurent G. Model of transient oscillatory synchronization in the locust antennal lobe. Neuron. 2001;30:553-567.
3. Friedrich R, Stopfer M. Recent dynamics in olfactory population coding. Curr Opin Neurobiol. 2001;11:468-474.
4. Laurent G, Stopfer M, Friedrich RW, Rabinovich MI, Volkovskii A, Abarbanel HD. Odor encoding as an active, dynamical process: experiments, computation, and theory. Annu Rev Neurosci. 2001;24:263-297.

*Joined NICHD in the fall of 2002

# BRIDGING THE GAP BETWEEN MONOGENIC TRAITS AND COMPLEX INHERITED DISORDERS OF CHILDHOOD

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Sara Stern-Nezer, *Guest Researcher*[a]
Mark Bochey, B.S., *Guest Researcher*[b]

The recently established Unit on Pediatric Genetics investigates several monogenic disorders. Our overarching goal is to improve the understanding, diagnosis, and treatment of these inherited diseases. A longer-term goal is the application of genetic approaches to common pediatric diseases; in these cases, associated molecular variations will provide the basis both for pathophysiological insights and tailored preventive strategies.

**Disorders of Copper Transport**
*Liu, Kaler; in collaboration with Goldstein, Holmes, Hoffman, Chen*
Menkes' disease is an X-linked recessive neurodegenerative disorder caused by defects in a gene that encodes an evolutionarily conserved copper-transporting ATPase. In mammals, the gene product functions as an intracellular pump to transport copper into *trans*-Golgi spaces for incorporation into copper-requiring enzymes; it also mediates copper exodus from cells. Our work on the disease includes the development of rapid and reliable neurochemical and molecular techniques for very early diagnosis. These efforts dovetail with our clinical trial of very early copper replacement therapy for affected infants. For many years, researchers in the field presumed that early identification and treatment with copper histidine were the key elements in successful management of Menkes' disease. However, the cumulative clinical, biochemical, and molecular findings from our study indicate that such is not true. Our results show that the blood-brain barrier poses a challenging obstacle in a majority of patients and suggest a molecular basis for treatment responsivity in the minority of patients with successful neurodevelopmental outcomes. Consequently, we are developing alternative therapeutic approaches (e.g., intrathecal copper injections).

To unravel the pathophysiological cascades associated with copper deficiency in the developing brain, we performed Affymetrix (oligonucleotide microarray) expression profiling studies utilizing postmortem tissues from several of our patients who died despite very early treatment. Their mutations predicted little or no functional copper transport activity. As expected, expression of the Menkes' disease gene was detected in control brains but not in the patient tissue. We detected approximately 350 dysregulated genes, including those involved in neuronal signalling pathways, synaptic function, mitochondrial function, and ribosomal function/translation and metabolism, and several ESTs of unknown function. A large share of up-regulated genes was involved in anti-apoptosis and stress response. These findings provide initial insight into the pathogenetic basis of neurodegeneration in Menkes patients with poor response to very early treatment with subcutaneous copper histidine injections.

### Molecular Basis of Bernard-Soulier Syndrome

*Kaler, in collaboration with Stern-Nezer*

The platelet membrane glycoprotein (GP) Ib-V-IX complex is the receptor for von Willebrand factor and is composed of four polypeptides: GPIb alpha, GPIb beta, GPIX, and GPV. A qualitative or quantitative deficiency in the complex causes the human platelet disorder Bernard-Soulier syndrome (BSS). Typically, BSS is an autosomal recessive disorder presenting with mild thrombocytopenia, circulating "giant" platelets, and a bleeding phenotype in infancy. Bleeding in BSS is more severe than would be predicted by platelet count and is explained by a defect in primary hemostasis. We identified a novel mutation at the GP 1b beta locus in an infant haplo-insufficient for the gene as a consequence of heterozygous deletion of chromosome 22q11 (velocardiofacial syndrome). Family studies and functional characterization of the mutant allele are under way with chinese hamster ovary (CHO) cells transfected with the GPIbα and GP IX genes. The primary investigations provide the basis for a new clinical protocol to evaluate other individuals with velocardiofacial syndrome (a relatively common genetic syndrome occurring in 1 out of 4,000 live births) for related abnormal-ities in platelet function.

### X Chromosome Inactivation and Developmental Anomalies

*Bochey, Kaler*

Nonrandom (or "skewed") X-chromosome inactivation has been implicated in the etiology of certain X-linked dominant traits (e.g., Rett syndrome). In such situations, female carriers of deleterious alleles on one X chromosome are spared disease manifestations due to favorably skewed X inactivation patterns; their female offspring (in whom X inactivation is random), however, are at risk for expression of the mutant allele. Prenatal lethality in male offspring who inherit the mutant allele explains the observed female predominance.

The constellation of birth defects (sternal cleft, abdominal raphe, and hemangiomas) shows a distinctive female predilection; available medical literature indicates that over 92 percent of cases of Rett syndrome occur in females. The same situation is seen in the related phenotype PHACE (posterior fossa brain malformations, hemangiomas, arterial anomalies, coarctation of the aorta and cardiac defects, and eye abnormalities). We documented skewed X inactivation in the mother of a PHACE patient, speculate that the phenotype represents an X-linked dominant trait that is lethal in males, and are exploring the hypothesis that defects in a transcription factor or other X chromosomal gene influencing development are responsible for this condition.

## SELECTED PUBLICATIONS

1. Devaney JM, Pettit EL, Kaler SG, Vallone PM, Butler JM, Marino MA. Genotyping of two mutations in the HFE gene using single base extension and high-performance liquid chromatography. Anal Chem. 2001;73:620-624.
2. Gasch AT, Caruso RC, Kaiser-Kupfer M, Kaler SG. Menkes Disease: expanded spectrum of ophthalmic findings in Menkes disease. Ophthalmology. 2002;109:1477-1483.
3. Kaler SG, Holmes CS, Goldstein DS. Perfect sensitivity and specificity of plasma catechol analyses for neonatal diagnosis of Menkes disease. Pediatr Res. 2002;51, Part 2,225A:1309.
4. Kaler SG, Liu P-C, Wattendorf DJ, Rosenbaum KN. Novel molecular mechanism for occipital horn syndrome. Am J Hum Genet. 2001;69, Suppl.609:2513.
5. Kaler SG, Ng D, Luban N, Schonberg S. Heterozygous 22q11 deletion and missense mutation in gpIb beta associated with velocardiofacial and Bernard-Soulier syndromes. Pediatr Res. 2001;49, Part 2:184A/1042.
6. Kaler SG. Menkes disease. eMedicine Journal 2001;2 (http://www.emedicine.com/ped/topic1417.htm).
7. Liu PC, Chen YW, Hoffman EP, Kaler SG. Expression profiling of Menkes disease brain. Am J Hum Genet. 2001;69(Suppl);600:2460.

8. Liu P-C, McAndrew PE, Kaler SG. Rapid and robust screening of the Menkes disease/occipital horn syndrome gene. Gen Test. 2002;6:255-260.

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# DEVELOPMENTAL GENOMICS

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Vanessa Baxendale, M.S., *Research Associate*
H. Claire Taylor, B.S., *Postbaccalaureate Fellow*
Andrew Aziz, *Student*

It is increasingly obvious that biological processes in living organisms are tightly regulated by a network of genes. The traditional "one gene–one pathway" approach is probably not appropriate for studying the complicated biological processes of development. Our research uses functional genomic approaches to study germ cell maturation and sexual development (normal and pathological states) to understand the intricate regulatory mechanism of proliferation and differentiation. In addition, given that many apparently sporadic diseases have been shown to have a genetic component, our studies of pseudotumor cerebri in a Turkish population have led to the identification of a genetic risk factor for this "sporadic."

## Functional Genomic Studies of Germ Cell Development in Spermatogenesis

*Pang, Wu, Baxendale, Taylor, Leung, Rennert, Chan; in collaboration with Dym, Ravindranath, Su, Stitely, Griffith, Rowe*

Spermatogenesis is a highly regulated process in which spermatogonial stem cells undergo mitotic expansion and differentiation. Distinct morphological and biological characteristics of germ cells at various developmental stages of spermatogenesis allow their isolation in relatively pure form. Animal models permit the study of "arrest and restart" of spermatogonial differentiation, providing a unique system for studying stem cells and the genetic factors that regulate their proliferation and differentiation.

One goal of our research is to delineate the genetic network that regulates spermatogenesis. Using cDNA microarrays, we are studying changes in gene expression in type A spermatogonia (Sg), pachytene spermatocytes (Sc), and round spermatids (Sd) of the mouse. We employed two types of microarrays, namely, Nylon-membrane-based Mouse GeneFilters® containing 5,184 mouse genes (ResGen®) and glass-slide microarrays printed with the NIA 15K mouse cDNA clone set. In the GeneFilters® experiments, we identified 79 differentially expressed genes and ESTs in the three types of germ cells. Quantitative real-time PCR confirmed the differential expression of a number of these genes. In the glass-slide microarray experiments, we studied the changes in gene expression in the transition from Sc to Sd. We identified 161 differentially expressed genes. More than one-quarter (43/161) were uncharacterized genes. A larger number of genes (110/161) were preferentially expressed in Sd. Functional categorization indicated that genes of signal transduction, energy metabolism, biosynthesis, and cellular transport were preferentially expressed in Sd while genes for chromatin remodeling were expressed only in Sc. Several testis-specific genes (feminization 1b homolog, phosphatidylcholine transfer protein-like, sperm-specific antigen 1, and cDNA moderately similar to casein kinase) were preferentially expressed in Sd. We achieved confirmation of the results by both quantitative real-time PCR and serial analysis of gene expression (SAGE).

We performed SAGE on Sc and Sd by using the I-SAGE kit (Invitrogen Corp). We sequenced 101,068 and 106,212 tags of the Sc and Sd library, respectively. Excluding singletons, they represented 10,717 and 10,135 genes, respectively. In the Sc library, four tags were present at ≥ 0.5 percent; they matched a mitochondrial sequence (0.65 percent), t-complex-associated testis 3 (0.57 percent), and Y box protein 2 (0.50 percent). The third most abundant tag (0.56 percent) had multiple hits in the SAGE map database. In the Sd library, four tags were present at ≥ 0.5 percent. The most abundant tag was protamine 2 (1.31 percent) followed by that matching FK506 binding protein (1.18 percent) and a tag that matched a mitochondrial sequence (0.64 percent). The fourth most abundant tag (0.56 percent) had multiple hits. Virtual subtraction of the two libraries yielded 4,344 Sc-specific tags and 4,155 Sd-specific tags. The majority of the cell stage–specific tags were present at less than five copies. We found that 353 Sc-specific tags were present at ≥ 5 copies and that only 38 of these were present at ≥ 10 copies. The corresponding figures for Sd-specific tags were 266 and 27. The most abundant Sc-specific tag matched Janus kinase 3 (43 tags, 0.04 percent of library) followed by that matching WW domain binding protein 4 (37 tags, 0.034 percent) and dynein (28 tags, 0.026 percent). Two of the three most abundant Sd-specific tags, CAGAAGGCGG and TATTAAAGCT, both at 18 copies (0.017 percent), were novel with no hit in the SAGE map database. The other tag present at 18 copies matched a RIKEN cDNA.

Comparison of the results obtained by cDNA microarray hybridization and SAGE indicated a high degree of concordance (≥80 percent) between the two methods. Discordance was limited to genes of low expression level. Our work identifies a large number of genes previously not known to be expressed in germ cells (characterized genes + ESTs ≥ 12,270) as well as novel genes. It is the first investigation to present a detailed comparison of the gene expression pattern in mouse germ cells. It provides the basis for investigation of genetic regulation of spermatogenesis and for the definition of these processes in pathological conditions.

| Genes | High in Sc | High in Sd |
|---|---|---|
| Protein Turnover | 4 | 6 |
| Cell Cycle Regulation | 3 | 4 |
| Apoptosis and Cell Proliferation | 5 | 6 |
| Signal Transduction | 2 | 17 |
| Energy Metabolism | 3 | 13 |
| Biosynthesis | 1 | 4 |
| Transcription Factors | 2 | 1 |
| Gene Expression Regulation | 3 | 5 |
| Chromatin Remodeling | 3 | 0 |
| Transport Proteins | 4 | 9 |
| Protein Quality Control System | 1 | 3 |
| Cellular Remodeling | 6 | 3 |
| Miscellaneous | 2 | 4 |
| Uncharacterized | 12 | 35 |

**TABLE 1**

Functional categorization of genes identified in the 15K mouse cDNA microarray experiment. Genes preferentially expressed in pachytene-specific cell type are highlighted.

### Physiological and Genetic Effects of Disease-Causing Mutations of the Luteinizing Hormone Receptor

*Leung, Wu, Aziz, Pang, Rennert, Chan; in collaboration with Leschek, Martin, Al-Muslim*

Constitutively activating mutations of the human luteinizing hormone/chorionic gonadotropin receptor (LHR) cause familial male-limited precocious puberty (FMPP), a non-central form of gonadotropin-independent precocious puberty. In collaboration with Dr. Malcolm Martin and Dr. Ellen Leschek, we have identified two FMPP patients who developed testicular neoplasia. To study the impact of constitutive activation of the LH/hCG signaling pathway on spermatogenesis and sexual development as well as the potential tumorigenic consequences of a constitutively activated LHR, we have generated an *in vitro* cell model and are in the process of generating a transgenic animal model. We transfected MA-10 cells with LHR carrying activating mutations and compared the cDNA microarray profile of expressed genes in cells expressing the mutated LHR with that of control cells. Preliminary studies of one mutated LHR indicated up-regulation of genes associated with cell proliferation and down-regulation of genes associated with differentiation. Interestingly, several genes known to be involved in spermatogenesis were down-regulated in cells expressing the mutated LHR. We are expanding this experimental approach with the transfection of different mutated LHR genes and an analysis employing more extensive mouse cDNA microarrays.

The antithesis of FMPP is Leydig cell hypoplasia (LCH). In LCH patients, mutation inactivates the LHR, resulting in reduced production of testosterone, which causes hypergonadotrophic hypogonadism or male pseudohermaphroditism. We recently identified a novel inactivating homozygous mutation in the LHR of a 19-year-old patient with male pseudohermaphroditism. The single base substitution T1505C caused replacement of Leu-502 by Pro in the transmembrane helix (TM) IV of LHR. This change disrupted the $\alpha$ helical structure of TM IV and resulted in the inactivation of the LHR. This is the first disease-causing mutation identified in TM IV of the LHR. The transfected mutant receptor is unable to trigger cAMP production following hCG stimulation. The role of TM IV in signal transduction of the LHR is not known. This mutation provides a tool with which to investigate the role of the TM IV in the active-inactive conformation transition of the receptor. It is known that cells process the mutated LHRs abnormally in either activated or inactivated form. To investigate the trafficking of mutated LHR *in vitro*, we have fused the coding sequence of green fluorescent protein (GFP) to that of wild-type and mutated LHR. We will study the trafficking of the fused protein by fluorescent microscopy. The information generated from this approach should enhance our knowledge on the cellular processing of the LHR.

The clinical impact of the activating mutation of LHR has always been considered to be limited to sexual development. The abnormal social behavior of patients was thought to be secondary to precocious sexual maturation. Expression of the LHR in the brain has been demonstrated. We speculate that the abnormal behavior of FMPP patients is caused by the expression of the mutated LHR in the brain. To examine this hypothesis, we plan to identify the cellular localization of LHR in the brain. To generate the transgenic animals, we are making constructs containing a 2 Kb 5'UTR putative promoter sequence of murine LHR and a GFP-coupled wild-type and mutated LHR for introduction into mouse ES cells. We will use the animal model to study the impact of constitutively activated LHR on spermatogenesis and sexual and neurological development.

### Identification of Genetic Risk Factors for Pseudotumor Cerebri in Turkish Population

*Dogulu, Leung, Baxendale, Rennert, Chan; in collaboration with Kansu, Ozguc*

Pseudotumor cerebri (PTC) is a syndrome characterized by symptoms and signs of isolated intracranial hypertension leading to catastrophic effects on the visual system in the absence of clinical, laboratory, or radiological evidence of a space-occupying lesion or hydrocephalus. The clinical picture is caused by increased cerebrospinal fluid (CSF) pressure; however, its pathogenesis is not well understood. The most prevalent hypothesis is that the increased CSF pressure is attributable to reduced CSF absorption through the arachnoid villi. Although the majority of cases are non-familial, reports of familial PTC raise the possibility of some genetic variations/defects that lead to clinical manifestations following exposure to a precipitating agent. We hypothesize that PTC is multifactorial and that an underlying genetic thrombotic risk factor predisposes the patients to develop local thrombi lining arachnoid villi, which in turn leads to increased intracranial pressure without demonstrable cerebral venous thrombosis by conventional imaging techniques. Such a genetic variation might occur in coagulation factor V.

Coagulation Factor V is an enzyme cofactor with pivotal functions in hemostasis. Several polymorphisms/mutations have been identified among the 25 exons of the Factor V gene. Altered activity of mutated Factor V is the most common hereditary blood coagulation disorder predisposing to thrombosis. A number of mutations/polymorphisms of the Factor V gene, including $V_{\text{Hong Kong}}$ (Arg306Gly), $V_{\text{Cambridge}}$ (Arg306Thr), Arg485Lys, $V_{\text{Leiden}}$ (Arg506Gln), and the R2 allele (Arg-1299), all known to be associated with thrombotic risk, are located in exons 7, 10, and13. We scanned exons 7 and 10 of Factor V gene for mutations in 51 PTC patients and 69 controls from Turkey. The G1628A single nucleotide substitution (Arg485Lys replacement) in exon 10 was previously associated with increased risk of coronary artery heart disease in the Chinese population (Le *et al.*, Clin Genet. 2000;57:296). It was found to be significantly associated with the development of PTC in the Turkish population. This is the first study to document a genetic risk factor for PTC. We are in the process of analyzing exon 13 of Factor V and the Factor II gene, which also is an important predisposing component of thrombosis development.

## SELECTED PUBLICATIONS

1. Bastain TM, Lewczyk CM, Sharp WS, James RS, Long RT, Eagen PB, Ebens CL, Meck JM, Chan WY, Sidransky ER, Rapoport JL, Castellanos FX. Cytogenetic abnormalities in attention-deficit/hyperactivity disorder. J Am Acad Child Adolesc Psychiatry. 2002;41:1-5.
2. Blomberg LA, Chan WY, Clerch L, Massaro G, Massaro D. Molecular cloning and characterization of two genes up-regulated early in lung development. Biochim Biophys Acta. 2002;1574:391-398.
3. Chan WY, Rennert OM. Molecular aspects of sexual differentiation. Curr Mol Med. 2002;2:25-37.
4. Leschek EW, Chan WY, Diamond D, Laefer M, Jones J, Barnes KM, Cutler GB, Jr. Nodular Leydig cell hyperplasia in a boy with familial male-limited precocious puberty (FMPP). J Pediatr. 2001;138:949-951.
5. Sandrini F, Farmakidis C, Kirschner LS, Wu SM, Tullio-Pelet A, Lyonnet S, Metzger DL, Bourdony CJ, Hochberg Z, Chan WY, Stratakis CA. Spectrum of mutations of the AAAS gene and genotype-phenotype correlation in patients with isolated resistance to corticotropin or Allgrove syndrome. J Clin Endocrinol Metab. 2001;86:5433-5437.
6. Wu SM, Leschek EW, Rennert OM, Chan WY. Luteinizing hormone receptor mutations in sexual development and cancer. J Pediat Path Mol Med. 2000;19:21-40.

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# GENETIC AND ENVIRONMENTAL DETERMINANTS OF PRIMATE BIOBEHAVIORAL DEVELOPMENT

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Our research involves broad-based investigation of primate biobehavioral development through comparative longitudinal studies of rhesus monkeys and other nonhuman primate species. Our primary research goals are to characterize different distinctive biobehavioral phenotypes in our rhesus monkey colony, to determine how genetic and environmental factors interact to shape this primate's development, and to assess the long-term biobehavioral consequences for monkeys from different genetic backgrounds when they are reared in various physical and social environments. A second major focus of our research involves how rhesus monkeys and other nonhuman primate species born and raised under different laboratory conditions adapt to placement into environments that model specific features of their natural habitat. In this program of research, we assess adaptation by examining behavioral repertoires and monitoring a variety of physiological systems in monkeys throughout the lifespan, developing broad-based indices of relative physical and psychological well-being. We also assess the responses of subjects to experimental manipulations of selected features of their respective environments. Whenever possible, we collect field data for appropriate comparisons. We also focus on investigating the cognitive, behavioral, and social processes involved in adaptation to new settings and circumstances.

## Developmental Continuity of Individual Differences in Rhesus Monkey Biobehavioral Development

*Champoux, Higley S, Novak, Parker, Roma, Shannon-Lindell, Suomi; in collaboration with Bennett, Graham*

Several of our ongoing studies focus on possible interactions between a polymorphism (due to length variation in the promoter region) in *5-HTT*, which is a candidate gene for impaired serotonergic function, and differential early social experience. This past year, we published the first report of a specific gene-environment interaction in a nonhuman primate species: rhesus monkeys with the "short" (*LS)* allele exhibited deficits in central serotonin metabolism compared with those with the "long" (*LL)* allele, but only if they had been nursery-reared; in contrast, *LS* monkeys reared by their biological mother had normal serotonin metabolism, suggesting a "buffering" effect of maternal rearing. A second study reported a similar buffering effect of maternal rearing for measures of state control and visual orienting obtained during the first month of life. Preliminary analyses have revealed additional examples of maternal buffering for *LS* rhesus monkeys in the expression of impulsive aggression and social play behavior during the monkeys' juvenile years.

Rhesus monkeys are notoriously aggressive as a species compared with other macaques and most other primates; Barbary macaques, on the other hand, are unusual in their relatively low levels of aggression. This past year, we were able to genotype members of a group of free-ranging Barbary macaques with respect to the *5-HTT* gene and, unlike the case for rhesus monkeys, found no individuals with either the LS or the LL allele. Instead, all the sampled Barbary macaques had an "extra long" (XL) allele, a form found in less than 2 percent of the rhesus monkeys genotyped to date. We are currently genotyping other Barbary macaques who came from different geographic regions than our original group to evaluate the generality of our finding. To model the evolutionary history of *5-HTT* in primates, we have also begun coding the promoter region of the *5-HTT* gene in several other nonhuman primate species representing different taxa.

This past year, we also characterized a polymorphism in the MAO-A receptor gene, demonstrated functional differences among the different alleles, genotyped the LCE rhesus monkey colony with respect to the above polymorphism, and began analyzing a variety of behavioral and physiological measures as a function of genetic status and early rearing history. Our initial analysis revealed a significant allele–rearing history interaction for aggressive behavior during the juvenile years: whereas mother-reared monkeys with the "7" allele initiated more bouts of aggression than those with the "5" or "6" allele, nursery-reared monkeys exhibited the reverse. Similar analyses are currently under way for other behavioral and physiological measures collected from the same monkeys throughout their development.

We also completed a study investigating the relationship between serotonin metabolism and measures of impulsive and aggressive behavior in adult female rhesus monkeys living in free-ranging naturalistic social groups. Previous work had demonstrated that low cerebrospinal fluid (CSF) concentrations of 5-HIAA, the primary central serotonin metabolite, were associated with a relatively high incidence of risky, impulsive behavior and socially inappropriate, violent aggression (but not with low-level, socially appropriate dominance behavior) in free-ranging adult male rhesus monkeys and in both adult male and female rhesus monkeys reared in captivity. Our analyses revealed that low CSF 5-HIAA concentrations in free-ranging females were also associated with excessive risky, impulsive behavior but not with violent aggression; instead, we found a significant negative correlation between CSF 5-HIAA and socially appropriate dominance behavior. The findings suggest a divergence between male and female rhesus monkeys in their competitive strategies, which may be explicable in terms of the distinctive social structure and life history patterns in the species.

### Behavioral Effects of Long-Chain Essential Fatty Acid Supplements

*Champoux, Shannon-Lindell, Suomi; in collaboration with Higley J, Hibbeln*

Finally, we published a study examining the biobehavioral consequences of adding a long-chain essential fatty acid supplement to the standard diet of nursery-reared rhesus monkey infants. The supplement effectively raised their serum levels to those characteristic of infants raised (and nursed) by their biological mothers. The standard nursery formula diet, like most commercially available human infant formula diets, is lacking in certain long-chain essential fatty acids, and monkey infants raised on the standard formula consistently have lower serum levels of long-chain essential fatty acids than their mother-reared counterparts. Nursery-reared infants receiving dietary supplements exhibited accelerated motor maturation and enhanced visual orientation capabilities during their first month of life compared with infants fed the standard formula, more closely resembling the patterns normally seen in mother-reared infants. A follow-up study examined heart rate variability in the monkeys when they were three years of age. Reduced heart rate variability has been associated with the development of aggressive and sociopathic behavior in human children and with vascular disease and congestive heart failure in adults. Monkeys that had received the formula supplement exhibited significantly greater heart rate variability than their counterparts that had received the standard formula during infancy, despite the fact that both groups had been maintained on a supplemented diet following weaning.

### Adaptation of Laboratory-Reared Monkeys to Field Environments

*DeVinney, Morin, O'Neill-Wagner, Robbins, Suomi, Turrio, Yang*

This past year, we completed the analysis of data collected on mother-offspring pairs of rhesus monkeys during the offsprings' first two years of life while residing in two free-ranging social groups at the field site at Cayo Santiago, PR. Our objective was to determine the degree to which individual differences in the monkeys' behavior were related to individual differences in a variety of psychophysiological, endocrine, and morphometric measures in both the offspring and their mothers. Following capture for veterinary examination, offspring showed stable individual differences in heart rate, plasma concentrations of cortisol and prolactin, and virtually all morphometric measures from their first to second year. Body mass index and plasma concentrations of cortisol and growth hormone in offspring and their mothers were unrelated when the offspring were yearlings but became significantly correlated by the time the offspring were two years old. Mothers with low cortisol levels punished their infants more, whereas mothers with high cortisol levels showed the highest rate of infant carrying. Offspring exhibiting the highest frequencies of agitated behavior throughout the study also exhibited the highest cortisol levels following capture. These findings suggest that mothers' morphological and physiological patterns are related to their offsprings' behavior and physiology via developmental experience as well as through genetic/intrauterine factors.

We have also been conducting a study of the relationships between measures of neurobiological functioning and various dimensions of maternal behavior in rhesus monkey mother-offspring pairs living in the species-normative social group maintained in the Laboratory's five-acre field enclosure. CSF samples were collected from mothers when their infants were three to five months of age and assayed for 5-HIAA and HVA (a major central dopamine metabolite); we then compared the resulting values with different aspects of maternal behavior recorded when their infants were one to three, four to six, and seven to nine months old, respectively. Measures of maternal protectiveness and infant restraint during the latter two time periods were negatively correlated with mothers' 5-HIAA and HVA concentrations. In contrast, measures of two forms of active infant rejection were positively correlated with concentrations of both monoamine metabolites during the latter two time periods, whereas the rate of passive rejections was not significantly related to concentrations of either metabolite during any time period.

Another study investigating mother-infant relationships in rhesus monkeys housed in the LCE field enclosure compared primiparous with multiparous females with respect to various aspects of their maternal behavior and the behavior of their infants. During their first month of life, offspring of multiparous females were more active than were primiparous infants. They initiated and broke contact with and approached and left their mothers more frequently than their primiparous counterparts. Throughout most of their infants' first five months of life, primiparous mothers exhibited higher rates of initiating ventral contact with their infants as well as higher rates of initiating nipple contact. These findings parallel parity differences in mother-infant relationships previously reported from field studies of rhesus monkeys and several other Old World monkey species.

We completed a study of continuity and change in patterns of adrenocortical functioning throughout the juvenile years in rhesus monkeys reared in three different physical and social environments for their first seven months of life but thereafter placed together and subsequently maintained in large peer groups. Monkeys from all three rearing conditions (mother-peer, surrogate-peer, and peer-only) exhibited significant increases in plasma cortisol over their first five months but then showed steady declines from one to three years of age; females consistently had higher concentrations than males at all ages. Plasma cortisol levels of surrogate-peer–reared subjects were lower than those of their mother-peer–reared counterparts (with peer-only–reared subjects intermediate) during the first month of life, but not at later ages. Juvenile cortisol levels were significantly correlated with infant levels in all samples collected after two weeks of age, indicating long-term developmental stability of individual differences. Saliva samples were collected from a subset of these subjects at one, two, and three years. Analysis of salivary cortisol concentrations from the subgroup revealed a significant age-related decline as well as lower levels for surrogate-peer–reared monkeys at each age, but no significant sex differences at any age. Comparison of salivary and plasma cortisol values obtained from the same subjects yielded a highly significant positive correlation.

## SELECTED PUBLICATIONS

1. Bennett AJ, Lesch KP, Heils A, Long JC, Lorenz JG, Shoaf SE, Champoux M, Suomi SJ, Linnoila MV, Higley JD. Early experience and serotonin transporter gene variation interact to influence primate CNS function. Mol Psychiatry. 2002;7:118-122.
2. Bennett AJ, Sponberg AC, Graham T, Lindell SG, Suomi SJ, Higley JD, DePetrillo PB. Initial alcohol exposure results in stress-dependent acute increases and subsequent decreases in CSF 5-HIAA concentrations and cardiac signal complexity in alcohol-naive rhesus monkeys. Eur J Pharmacol. 2001;433:169-172.
3. Byrne GD, Suomi SJ. Cortisol reactivity and its relation to homecage behavior and personality ratings in tufted capuchin juveniles from birth to six years of age. Psychoneuroendocrinol. 2002;27:139-154.
4. Champoux M, Bennett AJ, Shannon C, Higley JD, Lesch KP, Suomi SJ. Serotonin transporter gene polymorphism, differential early rearing, and behavior in rhesus monkey neonates. Mol Psychiatry. 2002;7:1058-1063.
5. Champoux M., Hibbeln J, Shannon C, Majchrzak S, Suomi SJ, Salem NT, Higley JD. Essential fatty acid formula supplementation and neuromotor development in rhesus monkey neonates. Pediatr Res. 2002;51:273-281.
6. Fahlke C, Garpenstrand H, Oreland L, Suomi SJ, Higley JD. Platelet monoamine oxidase activity in a nonhuman primate model of type 2 excessive alcohol consumption. Am J Psychiatry 2002;159:2107.
7. Gerald MS, Higley S, Lussier ID, Westergaard GC, Suomi SJ, Higley JD. Variation in reproductive outcomes for captive male rhesus macaques differing in CSF 5-hydroxyindoleacetic acid concentrations. Brain Behav Evol. 2002;60:117-124.
8. Strome EM, Higley JD, Loriaux DL, Suomi SJ, Doudet DJ, Wheler GHT. Intracerebroventricular corticotropin-releasing factor has behavioral effects in non-human primates depending on the social context, and increases cerebral glucose metabolism in limbic regions. Proc Nat Acad Sci USA. 2002;99:15749-15754.
9. Suomi SJ. Harry Frederick Harlow (1905-1981). In: Smelser NJ, Baltes PB, eds. International encyclopedia of the social and behavioral sciences. Amsterdam: Pergamon 2002;6473-6476.
10. Suomi SJ. How gene-environment interactions can influence emotional development in rhesus monkeys. In: Garcia-Coll C, Bearer EL, Lerner RM, eds. Nature and nurture: the complex interplay of genetic and environmental influences on human development. Mahwah, NJ: Erlbaum;in press.
11. Suomi SJ. Parents, peers, and the process of socialization in primates. In: Borkowski JG, Ramey SL, Bristol-Power M, eds. Parenting and the child's world: influences on academic, intellectual, and social-emotional development. Mahwah, NJ: Erlbaum, 2002;265-279.

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# CHILD AND FAMILY DEVELOPMENT IN THE FIRST TWO DECADES OF LIFE

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The Child and Family Research Section (CFRS) investigates dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in human beings during the early years of the life course. Our overall goals are to describe, analyze, and assess the capabilities and proclivities of developing children, including their genetic characteristics, physiological functioning, perceptual and cognitive abilities, and emotional, social, and interactional styles as well as the nature and consequences for children and parents of family development and children's exposure to and interactions with the inanimate environment. Project designs are longitudinal, cross-sectional, and cross-cultural. Sociodemographic comparisons include family socioeconomic status, maternal age and employment status, and child parity and daycare experience. Study sites include Argentina, Australia, Belgium, Brazil, Cameroon, Canada, England, France, Israel, Italy, Japan, Kenya, and Korea as well as the United States; the section pursues cross-cultural as well as intra-cultural comparisons of human development.

## Parenting and Child Development

*Bornstein, Suwalsky, Hahn, Haynes, Hendricks, Painter*

Socioeconomic status (SES) is conceived of as a multidimensional construct that is indexed by quantitative factors associated with parents' educational achievement, occupational status, and financial income. In a multilevel study of European American families of diverse SES, we used structural equation modeling to explore direct and indirect relations of SES for multiple indexes of maternal parenting and multiple indicators of infant development. We evaluated the predictive validity of SES as a composite as well as its several components alone. Although each component of SES predicted some aspects of maternal and infant behavior, maternal education proved the most robust unique predictor of SES effects, separate too from maternal intelligence and personality.

In a separate study of parenting effects on child language, we obtained data on children's language through biweekly interviews with mothers. From these interviews, we calculated the timing of language milestones in children (e.g., 50 words in productive language, combinatorial speech). To analyze the effects of maternal verbal responsiveness on the timing of language achievements, we used Events History Analysis, a statistical approach that is suited to addressing questions of whether and by how much predictors affect the timing of events. First words in production, 50 words in receptive language, and maternal responsiveness at nine and 13 months were each significant predictors of the timing of 50 words in production. The relation between earlier responsiveness and the timing of 50 words is mediated by intervening linguistic competencies as well as by mothers' later responsiveness. In support of this conclusion, our findings demonstrate that nine-month responsiveness predicted the timing of 50 words in receptive language along with the timing of first words in production and 13-month responsiveness. In turn, these mediators predicted when children acquired 50 words in their productive lexicons. In contrast, responsiveness at 13 months contributed unique variance to the timing of 50 words in production over and above the timing of first words in production, the timing of 50 words in receptive language, and responsiveness at nine months. With respect to the timing of children's achievement of combinatorial speech, responsiveness at nine months contributed unique variance, above the contribution of 50 words in receptive language, but did not contribute unique variance above the timing of first words in production or responsiveness at 13 months. Responsiveness at 13 months contributed unique variance to the timing of combinatorial speech over and above the timing of first words in production, the timing of 50 words in receptive language, and responsiveness at nine months. Again, responsiveness at nine months improved model fit through its relation with first words in production and responsiveness at 13 months, both of which uniquely predicted when children first combine words. Children with verbally responsive mothers achieve the vocabulary spurt and combine words into simple sentences sooner in development than children with less responsive mothers. Predictive associations between responsiveness and the timing of children's language milestone are more robust at 13 than at nine months, and they matter above the contributions of children's earlier language abilities. The best-fitting model, however, is one that includes both children's and mothers' contributions to second-year language gains. That is, a child who produces first words sooner in development, coupled with a verbally responsive mother, is at a strong advantage for precocious achievement of key language milestones.

### Family and Child Acculturation in Modern America

*Bornstein; in collaboration with Cote*

One study examined similarities and differences in mothers' and infants' activities and interactions among Japanese American and South American immigrant dyads when infants were five months of age. Few relations between maternal acculturation level or individualism/collectivism and maternal parenting or infant behaviors emerged in either group. However, we observed group differences in mothers' and infants' behaviors, indicating that mothers' culture of origin continues to influence parenting behavior in the two acculturating groups. We next examined cultural generality and specificity in relations among and between mothers' and infants' behaviors. Few relations among mothers' behaviors emerged, except for that between mothers' social behavior and other types of maternal behavior, which appear to reflect a common collectivist orientation of the two cultural groups. Few relations among infants' behaviors emerged, suggesting independence and plasticity in infant behavioral organization. Several expected relations between mothers' and infants' behaviors also emerged, pointing to some universal characteristics in mother-infant interactions.

A follow-up longitudinal study evaluated differences, continuity, and stability in cultural cognitions (acculturation, individualism, collectivism) and parenting cognitions (attributions, self-perceptions, knowledge) in the same samples of Japanese American and South American acculturating mothers when their children were five and 20 months of age. South American immigrant mothers were more collectivist than Japanese American immigrant mothers. Cultural group and attribution differences emerged for mothers' attributions in successful situations, whereas child age and attribution differences emerged for attributions in unsuccessful situations. Japanese American immigrant mothers' feelings of competence increased over time. South American immigrant mothers were more satisfied with the parenting role than Japanese immigrant mothers. Mothers' knowledge of parenting increased over time in both groups. Mothers' cultural cognitions were largely stable, and Japanese American mothers' parenting cognitions were highly stable. Further analysis evaluated prediction and coherence among mothers' cultural and parenting cognitions. Mothers' cultural cognitions at five months predicted some parenting cognitions at 20 months, particularly among Japanese American immigrant mothers. At five and 20 months, coherence among mothers' attributions was found for both cultural groups and among Japanese American mothers' perceptions of parenting. Even though we found a few relations across types of parenting cognitions, these domains of parenting cognitions appeared to be relatively independent. The study provides insight into the nature and structure of parenting cognitions in two groups acculturating to the United States.

## Infant and Child Development

*Bornstein, Suwalsky, Hahn, Haynes, Hendricks, Painter*

We conducted several studies geared to understanding physiology, perception, and cognition in infants leading to language development. First, we measured and quantified fetal cardiac function at 24, 30, and 36 weeks' gestation in terms of heart rate, variability, and episodic accelerations and later evaluated children's language capacity at 27 months. Thirty- and 36-week-old fetuses that displayed greater heart-rate variability and more episodic accelerations and fetuses that exhibited a more precipitous increase in heart-rate variability and acceleration over gestation achieved higher levels of language competence. Cardiac patterning during gestation appears to reflect an underlying neural substrate that persists through early childhood; individual variation in rate of development could be stable, or efficient cardiac function could positively influence the underlying neural substrate to enhance cognitive performance.

We next investigated infants' categorization of nonverbal objects based on static versus dynamic attributes of stimuli in four experiments: six-month-olds categorized static color images of animals and vehicles; six-month-olds categorized dynamic point-light displays showing only motions of the same animals and vehicles; six- and nine-month-olds were tested in a habituation-transfer paradigm, with half of the infants at each age habituating to static images and tested with dynamic point-light displays and the other half to dynamic point-light displays and tested with static images. Six-month-olds did not transfer. Only nine-month-olds who were habituated to dynamic displays showed evidence of category transfer to static images.

Further study compared naturalistic samples of four features of language in two-year-olds in the home in three contrasting situations: the child observed playing by her- or himself with mother nearby, the child and mother observed in direct play interaction, and the child and mother unobserved at a time the mother judged would provide a sample of the child's "optimal" language. Children produced more utterances and word roots and expressed themselves in longer utterances when in interaction than when playing "alone," but children's utterances, word roots, and utterance length were greatest in the "optimal" language production situation. Girls used more word roots and spoke with longer mean length of utterance (especially in the "optimal" language situation) than boys. Despite mean level differences, children maintained their rank orders across the three situations in use of word roots and in utterance length. These findings have implications for understanding children's language and the validity of sampling child language.

In a further, methodological examination of child language, children participated in three longitudinal studies of general language performance (cumulatively from one year, one month to six years, 10 months). Data were drawn from maternal questionnaires, maternal interviews, experimenter assessments, and teacher reports. We assessed general language performance at each age and the stability of individual differences across age in girls and boys separately and together. Across age, including the important transition from preschool to school, across multiple tests at each age, and across multiple reporters, children showed moderate to strong stability of individual differences; girls and boys alike were stable. In the second through fifth years, but not before or after, girls consistently outperformed boys in general language ability.

## SELECTED PUBLICATIONS*

1. Arterberry ME, Bornstein M. Infant perceptual and conceptual categorization: the roles of static and dynamic stimulus attributes. Cognition. 2002;86:1-24.
2. Arterberry ME, Bornstein MH. Three-month-old infants' categorization of animals and vehicles based on static and dynamic attributes. J Exp Child Psychol. 2001;80:333-346.
3. Bornstein M. Handbook of parenting. Bornstein MH, ed. Mahwah, NJ: Lawrence Erlbaum Associates, 2002; volumes 1-5.
4. Bornstein MH. Some questions for a science of "culture and parenting" (... but certainly not all). Int Soc Study Behav Dev Newsl. 2001;1:1-4.
5. Bornstein M, Bradley RH. Socioeconomic status, parenting, and child development. Bornstein MH, Bradley RH, eds. Mahwah, NJ: Lawrence Erlbaum Associates, 2002.
6. Bornstein M, DiPietro JA, Hahn C, Painter KM, Haynes O, Costigan KA. Prenatal cardiac function and postnatal cognitive development: an exploratory study. Infancy 2002;3:475-494.
7. Bornstein MH, Cote LR. Mother-infant interaction and acculturation I: behavioral comparisons in Japanese American and South American families. Int J Behav Dev. 2001;25:549-563.
8. Collins WA, Maccoby EE, Steinberg L, Hetherington EM, Bornstein M. Contemporary research on parenting: the case for nature and nurture. Am Psychol. 2002;55:218-232.
9. Cote LR, Bornstein MH. Mother-infant interaction and acculturation II: behavioral coherence and correspondence in Japanese American and South American families. Int J Behav Dev. 2001;25:564-576.
10. Lamb ME, Bornstein M, Teti D. Development in infancy: an introduction. Mahwah, NJ: Lawrence Erlbaum Associates, 2002.
11. Tamis-LeMonda CS, Bornstein M. Maternal responsiveness and early language acquisition. In: Kail RV, Reese HW, eds. Advances in child development and behavior. New York, NY: Academic Press, 2002;29:38.
12. Tamis-LeMonda CS, Bornstein MH. Baumwell L. Maternal responsiveness and children's achievement of language milestones. Child Dev. 2001;72:748-767.

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# CHILD DEVELOPMENT IN CONTEXT

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The primary theme underlying our research is that all developmental processes are powerfully influenced by their social and physical context. As a result, researchers must examine the interface between endogenous and exogenous processes, children's conceptions and perceptions of their experiences, and the ways in which knowledge of developmental processes can inform social policies and practices.

### Facilitating Children's Accounts of Experienced Events

*Lamb, Aldridge, Cederborg, Abbott, Esplin, Guterman, Orbach, Pipe, Roberts, Sternberg, Thierry*

One major program of research has involved the development and assessment of techniques for enhancing the informativeness of child witnesses and for evaluating the credibility of their accounts. Most studies in this research program focus on the relationship between interviewer style and the quality of information provided by young children. Several studies have confirmed that open-ended questions elicit longer and more detailed responses than more focused questions. Information elicited in response to open-ended prompts of recall memory is also more likely to be accurate in both analog and forensic contexts, and such findings have strengthened the generalizability of the results obtained in many laboratory studies.

In research conducted in collaboration with investigative agencies in the United States, United Kingdom, and Israel, we have shown that interviewers can increase the length and richness of children's accounts, regardless of the children's ages, by following protocols we designed to probe recall memory and reduce the reliance on more focused questions, which are more likely to elicit erroneous information. Use of the NICHD protocol dramatically increases the amount of information retrieved from four- to 13-year-old alleged victims when using open-ended prompts. Although preschoolers are often deemed incapable of responding to open-ended prompts, our recent research shows that similar proportions of details can be elicited by using open-ended prompts from children as young as four years and as old as 13 years. There are important age differences in the types of information that children provide, however. For example, ongoing analyses of interviews show steady increases with age in the amount of information children provide about the timing of events.

Adaptations of the NICHD Investigative Interview Protocol for use with young witnesses and alleged perpetrators are currently being used in the field as well. Preliminary analyses show that young witnesses recall as much information in total, as well as in response to open-ended prompts, as alleged victims do. Suspects tend to be more reluctant, but those who agree to talk provide as much information about their experiences as age mates who are alleged victims. Other field experiments have shown that mental context reinstatement and the introduction of gender-neutral anatomical drawings in the context of protocol-guided interviews also help children provide substantial numbers of additional details about alleged incidents of abuse.

In other ongoing research, we are exploring the extent to which use of the protocol facilitates decisions and interventions designed to prosecute offenders and protect children. Because many children do not disclose suspected abuse when interviewed, however, we are also exploring the characteristics of cases in which children do and do not make allegations or make allegations only reluctantly. We hope to understand the factors that prevent children from reporting abuse that they actually experienced. These studies, too, should help us develop procedures that can be implemented nonsuggestively in forensic settings in order to enhance the sensitivity and specificity of conclusions drawn from investigative interviews.

Another program of research is concerned with the effects of child and spouse abuse on the development of children and adolescents. Independent interviews with mothers, fathers, and children revealed widely divergent accounts of the families' histories of violence, and these differences complicate efforts to identify links between experiences and outcomes. In both middle childhood and adolescence, however, family violence appears to affect the offsprings' views of their parents. Children/adolescents felt less closely attached to the parents who had abused them, whereas spouse abuse had no apparent effects on the children's attachments to their parents.

### Adaptation to Nonparental Child Care

*Lamb, Abbott, Ahnert, Chuang, Hwang*

Another program of research has involved short- and long-term longitudinal studies in Göteborg (Sweden) and Berlin (Germany) of children who have different child care experiences. The longitudinal study in Sweden was initially designed to elucidate the effects of early care arrangements on the development of 145 children recruited in 1982 at an average age of 16 months. Initial analyses indicated that the quality of home care and the quality of alternative care had substantial effects on the children's verbal abilities, social skills, and personal maturity. These effects diminished as the children moved into the formal educational system and their individual personalities came to affect the adjustment to school. Preliminary analyses revealed no apparent effects of contrasting early care patterns on educational histories and the psychological status of these children at 15 years of age.

Longitudinal analyses revealed substantial stability over time in the children's personality styles. Of the "Big Five" personality factors, conscientiousness was coherent from toddlerhood, whereas the internal reliabilities of extroversion, agreeableness, neuroticism, and openness to experience increased over time. Scores on most of these factors were fairly stable over time, but children became less extroverted, more agreeable, and more conscientious with age. Although individual differences were stable, the children also became more ego-controlled over time. Boys' levels of ego resiliency were more stable over time than girls'; boys became less resilient from middle childhood into midadolescence, whereas girls became more ego-resilient as they entered adolescence.

In the Berlin longitudinal study, we have been measuring the psychophysiological, socioemotional, and behavioral tendencies of infants so that we can assess the effects of prior individual differences in behavioral and psychophysiological reactivity and infant-mother attachment on the adaptation to out-of-home center care. During an adaptation phase, in which mothers remained in the centers with their toddlers, securely attached infants had markedly lower cortisol levels than insecure infants. When the mothers stopped remaining with their infants, the cortisol responses of the securely attached toddlers were much more dramatic than the responses of the insecurely attached toddlers: on the initial separation days, cortisol levels rose over the first 60 minutes after arrival to levels twice as high as at home. Secure toddlers also fussed/cried upon separation more than insecurely attached toddlers. Cortisol and behavioral markers of distress were correlated in securely attached but not in insecurely attached toddlers. The security of attachment changed in many cases following the onset of child care, but attachments were more likely to become or remain secure when mothers remained longer in the child care facilities with their toddlers. Close examination of individual differences in cardiac reactivity and of the formation of relationships with care providers is now under way.

### Subcultural Variations in the Nature of Children's Early Experiences

*Lamb, Ahnert, Fouts, Hewlett, Rooparine*

Another project has focused on the description of early interaction in diverse ecological contexts. In each case, extended observations have been conducted to ensure the reliable measurement of individual differences. Comparable day-long observations of parents and infants in Quebec, Germany, the Central African Republic, Costa Rica, and Colombia, as well as in African-American and Euro-American families in the United States, are under way to explore further the effects of culture and context on early interactions. Extended observations of two- to five-year-old Bofi infants whose families either lived in villages or pursued a nomadic hunting and gathering lifestyle revealed that, contrary to widespread beliefs, weaning was seldom a time of parent-offspring conflict. In both groups, the children initiated weaning as they began to eat a variety of foodstuffs, although villagers were more likely to terminate breastfeeding at a predetermined time, whereas foragers let the children set the pace. Weaning was usually associated with pregnancy, and the children's reactions varied depending on the availability of additional care providers and maternal sensitivity.

### Subcultural Variations in Parental and Filial Perceptions and Beliefs

*Lamb, Bassen, Chuang, Fouts, Hwang; in collaboration with Cabrera*

We have also been investigating ways in which variations among rearing environments (especially as indexed by parental beliefs, values, and practices) affect children's development. In one line of research, gender differences in the self-perceptions of two cohorts of seventh to twelfth graders are undergoing longitudinal assessment in order to explore the antecedents and correlates of different styles of self-perception in adolescence. Rating themselves in 11 different roles, girls perceived themselves as more affiliative and less negatively affiliative in many roles than did boys. However, gender differences in assertion were not reliable, and girls' assertiveness did not decline over time. These results contrast with popular claims regarding girls' "loss of voice" in adolescence. Gender differences were context-specific and were more pronounced in ratings of "myself" as a boy/girl and "myself" with a close same-sex peer. To explore antecedents of these gender differences further, portions of the self-perception battery were completed by a group of Swedish 15-year-olds whose development has been documented systematically since infancy. Analyses of these data are currently under way.

## PUBLICATIONS

1. Ahnert L, Lamb, ME. The East German child care system: associations with caretaking and caretaking beliefs, children's early attachment and adjustment. Am Behav Scientist. 2001;44:1843-1863.

2. Fouts, HN. Central African families: a comparison of Bofi farmer and forager families. In: Roopnarine JL, Gielen UP, eds. Families in global perspective. Boston: Allyn & Bacon, in press.
3. Fouts HN. Social contexts of weaning: the importance of cross-cultural studies. In: Gielen UP, Roopnarine JL, eds. Childhood and adolescence in cross-cultural perspective. Westport CT: Greenwood Publishing Group, in press.
4. Gobbo C, Mega C, Pipe M-E. Does the nature of the experience influence children's suggestibility? A study of children's event memory. J Exp Child Psychol. 2002;81:502-530.
5. Hewlett BS, Lamb ME. Integrating evolution, culture and developmental psychology: explaining caregiver-infant proximity and responsiveness in Central Africa and the USA. In: Keller H, Poortinga YH, Schölmerich A, eds. Between culture and biology: perspectives on ontogenetic development. New York: Cambridge University Press, 2002;241-269.
6. Jones CH, Pipe M-E. How quickly do children forget events? A systematic study of children's event reports as a function of delay. Appl Cognit Psychol. 2002;16:1-14.
7. Lamb ME. Infant-father attachments and their impact on child development. In: Tamis-LeMonda CS, Cabrera N, eds. Handbook of father involvement: multidisciplinary perspectives. Mahwah, NJ: Erlbaum, 2002;93-117.
8. Lamb ME. Noncustodial fathers and their children. In: Tamis-LeMonda CS, Cabrera N, eds. Handbook of father involvement: multidisciplinary perspectives. Mahwah, NJ: Erlbaum, 2002;169-184.
9. Lamb ME, Ahnert L. [Institutional care contexts and their developmental relevance to young children]. In: Keller H, ed. Handbook of child development (3rd edition). Bern: Huber, in press [German].
10. Lamb ME, Bornstein MH, Teti DM. Development in infancy (4th edition). Mahwah, NJ: Erlbaum, 2002.
11. Lamb ME, Chuang SS, Hwang CP. Internal reliability, temporal stability, and correlates of individual differences in paternal involvement: a 15-year longitudinal study in Sweden. In: Day RD, Lamb ME, eds. Re-conceptualizing and measuring father involvement. Mahwah, NJ: Erlbaum, in press.
12. Lamb ME, Chuang SS, Wessels H, Broberg AG, Hwang CP. Emergence and construct validation of the big five factors in early childhood: a longitudinal analysis of their ontogeny in Sweden. Child Dev. 2002;73:1517-1524.
13. Lamb ME, Sternberg KJ, Orbach Y, Hershkowitz I, Horowitz D, Esplin PW. The effects of intensive training and ongoing supervision on the quality of investigative interviews with alleged sex abuse victims. Appl Dev Sci. 2002;6:114-125.
14. Leyendecker BL, Harwood RL, Lamb ME, Schölmerich A. Mothers' socialization goals and evaluations of desirable and undesirable everyday situations in two diverse cultural groups. Int J Behav Dev. 2002;26:248-258.
15. Orbach Y, Lamb ME, Sternberg KJ, Williams JMG, Dawud-Noursi S. The effect of being a victim or witness of family violence on the retrieval of autobiographical memories. Child Abuse Negl. 2001;25:1427-1437.
16. Pipe M-E, Salmon K. What children bring to the interview context: individual differences in children's event reports. In: Eisen ML, Quas JA, Goodman GS, eds. Memory and suggestibility in the forensic interview. Mahwah, NJ: Erlbaum, 2002;235-261.
17. Thierry KL, Spence MJ. Source-monitoring training facilitates preschoolers' eyewitness memory performance. Dev Psychol. 2002;38:428-437.

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# MECHANISMS REGULATING AUDITORY COMMUNICATION AND SOCIAL BEHAVIOR IN PRIMATES AND OTHER MAMMALS

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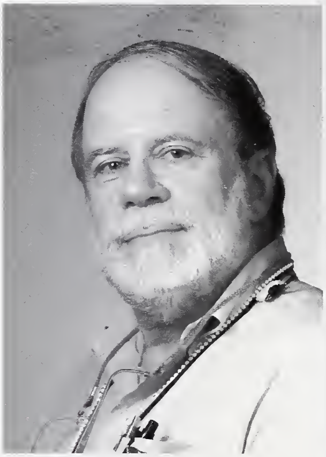

Our principal research goal is to understand the neural and hormonal mechanisms mediating expression of vocalizations and appropriate social responses to such sounds. In addition, we are attempting to understand the role of certain peptide hormones in the brain on the establishment and maintenance of social bonds between adults and between caregivers and their offspring.

## Acoustic Dimensions Underlying Nonhuman Primate Conversational Communication

*Soltis, Newman, Bernhards*

Using standard playback methodology, we completed a new study of vocal communication in adult female squirrel monkeys (*Saimiri sciureus*). The purpose of the study was to determine which acoustic component of a frequent vocalization, the "chuck," provided the most salient information regarding individual identity of the vocalizer. For our response measure, we used a chuck "response" by a group member to the playback stimulus within four seconds. We digitized chucks recorded from the test group and then presented them intact, with a frequency-modulated component referred to as the "flag" removed, or with the flag of another individual (from another social group) electronically spliced onto a chuck instead of the naturally occurring flag for that vocalization. The rate of response to intact chucks was significantly higher than to the same chucks with the flag removed or to chucks with unfamiliar flags spliced onto them. The results suggest that the flag is an important acoustic feature used by females to determine whether they vocally respond in this form of conversational communication. To test whether the acoustic information in flags was sufficient to for a statistically separation of vocalizers, we measured and entered into a discriminant analysis several parameters of flags (such as flag duration, peak frequency, and so forth). The results indicated that flag parameters alone were sufficient to separate the chucks of vocalizers in a social group.

## Hormonal Correlates of Affiliative Behavior in Nonhuman Primates

*Soltis, Newman, Bernhards*

In a second project, we are examining the hormonal correlates of affiliative behaviors in squirrel monkeys. Squirrel monkeys engage in three types of affiliation: mothering, allomothering by other adult females that extends to nursing and carrying, and same or opposite-sex affiliation among adults or adults and juveniles. Most of the affiliations involve adult females, as adult males are more solitary. We are investigating cortisol, which is implicated in the stress response, and prolactin, which has been implicated in affiliative behavior. We expected prolactin to be associated with allomothering because of its clear role in lactation and speculated that it may be associated with same-sex adult friendships because it has also been implicated in affiliative behaviors that do not involve lactation, such as male parenting behavior in the common marmoset, another neotropical primate. We compared prolactin and cortisol levels in blood and urine samples from the same monkey and noted a positive correlation. However, given that detection of prolactin requires a larger volume of urine than is typically produced in a single void, we collected urine samples from individual monkeys at the same time of day over several days and combined them for analysis. Despite the lack of precise temporal information, the advantage of using a noninvasive method for collecting samples for hormone analysis outweighs the disadvantage.

Based on analysis of data from three social groups, we concluded that urinary prolactin is positively associated with physical contact with infants and with the intensity of caregiving toward infants. Urinary cortisol is positively associated with the level of social disruption brought on by the presence of older infants and juveniles that spend a large portion of their day engaged in social play.

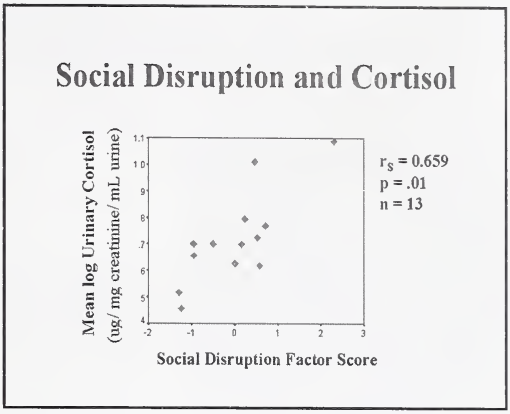

**FIGURE 19**

Positive correlation between urinary cortisol in adult females and the amount of disruptive playing by juveniles in social groups of captive squirrel monkeys. According to a principal components analysis, individual adults scoring high on this behavioral factor experienced a large number of play attempts by infants and juveniles and showed retaliatory aggression against them; they exhibited low levels of social affiliation with other adults. The relationship between individual factor scores and mean cortisol values is positive, suggesting that such disruption produces measurable physiological stress in the adult monkeys.

### Neuroethology of Infant Crying

*Newman, Bernhards*

Two new studies initiated this year aim to understand the mechanisms underlying infant crying and the response of potential caregivers to cry sounds. The first study uses immunocytochemical techniques to reveal brain sites at which the protein product (Fos) of the immediate-early (IE) gene *c-fos* is found following a bout of crying in infant rats and infant marmosets. We have refined our technique to a point where we can routinely demonstrate Fos-like activity in the brains of experimental subjects and will further refine the methods to permit us to distinguish between specific and nonspecific activity at brain sites. Eventually, we intend to use the same technique to identify brain sites where c-fos activity is associated with hearing infant cries. The second study uses playback techniques similar to those used in the "chuck" study (see above) to document response tendencies of adult squirrel monkeys upon hearing isolation calls. Initially, we are measuring the vocal responses of adults to adult isolation calls, but eventually we will measure the responses to infant cries. A major goal of our work is to identify the acoustic parameters in isolation calls ("cries") that promote vocal and nonvocal responses to these sounds. Data collection is still in progress.

## SELECTED PUBLICATIONS

1. Guard HJ, Newman JD, Roberts, RL. Morphine administration selectively facilitates social play in common marmosets. Dev Psychobiol. 2002;41:37-49.
2. Lorberbaum JP, Newman JD, Horwitz AR, Dubno JR, Lydiard RB, Hamner MB, Bohning DE, George MS. A potential role for thalamocingulate circuitry in human maternal behavior. Biol Psychiatry. 2002;51:431-445.

3. Newman JD. Auditory communication and central auditory mechanisms in the squirrel monkey: past and present. In: Ghazanfar A, ed. Primate audition: ethology and neurobiology. Boca Raton, FL: CRC Press LLC, 2002;227-246.
4. Soltis J, Bernhards D, Donkin H, Newman JD. The squirrel monkey chuck call: vocal response to playback chucks based on acoustic structure and affiliative relationship with the caller. Am J Primatol. 2002;57:119-130.

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# VACCINES AGAINST HUMAN PATHOGENIC BACTERIA

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## Search for a Polysaccharide Vaccine against *Mycobacterium tuberculosis*

*Dai, Zhang, Schneerson, Robbins; in collaboration with Morris, Muller, Collins, Schulz*

Tuberculosis remains a serious and common disease throughout the world. Worldwide emergence of multi-antibiotic–resistant strains, accelerated by the passage of *M. tuberculosis* in patients with AIDS, poses a public health problem. Despite the absence of evidence demonstrating that the BCG vaccine prevents primary pulmonary tuberculosis (the most common disease caused by *M. tuberculosis*), BCG remains the most frequently used vaccine in the world. The protective effect of BCG vaccination against meningitis in children has not been related to an antigen/s or a host immune component.

Research into a new vaccine is based on the similarity of primary infection caused by *Mycobacterium tuberculosis* with that of capsulated bacterial respiratory pathogens, viz: tuberculous meningitis has a similar age distribution as meningitis caused by meningococci, pneumococci, and *Haemophilus influenzae* type b; *M. tuberculosis* and other mycobacteria have a polysaccharide (PS) capsule *in vitro* and *in vivo;* and protein components prolonged the survival of but do not protect animals against challenge with wild-type *M. tuberculosis*. We purified a glucan and an arabinomannan from a saline extract of *M. tuberculosis*. The PSs were conjugated to *P. aeruginosa* recombinant protein A and shown to be immunogenic in mice and rabbits. Antibodies induced by these conjugates, whether actively induced or passively administered, did not protect mice against pulmonary challenge with *M. tuberculosis*. Antibodies to both PSs in adults, measured by ELISA, showed that patients with tuberculosis had much higher levels than did normal individuals. A standardized and quantitative assay for diagnosis of tuberculosis with these PS antigens is under way. In collaboration with CBER (FDA), both the glucan and arabinomannan conjugates are undergoing evaluation for their protective activity in mice.

## Vaccines against Enteric Pathogens

*Szu, Bryla, Li, Kossaczka, Cui, Hunt, Nguyen, Schneerson, Robbins; in collaboration with Shiloach, Lin, Bohach, Parke, Ahmed*

Surface PSs of Gram-negative enteric pathogens, in the form of capsule (CPS) or lipopolysaccharide (LPS), are both essential virulence factors and protective antigens. We enhanced the immunogenicity of these PSs binding to carrier proteins. Sequential clinical studies in adults and in children in Vietnam, an area with a high attack rate of typhoid fever, showed that the capsular PS (Vi) of *Salmonella typhi* conjugated to the recombinant

*Pseudomonas aeruginosa* exoprotein A (rEPA) elicited high responses against *S. typhi*. In a Phase III trial, about 12,000 two- to five-year-old children injected with the conjugate vaccine showed no significant side reaction. The efficacy of Vi-rEPA was 91 percent after 27 months of active surveillance and 88 percent after 28 to 40 months of passive surveillance. A conjugate vaccine against *Salmonella paratyphi* A, the second most common cause of enteric fever in Southeast Asia, was found to be safe and immunogenic in adults, teenagers, and then in two- to four-year-old children.

*Escherichia coli* O157, an emerging pathogen, causes hemolytic uremic syndrome in young children. A Phase I study of an O157 O-specific PS-rEPA conjugate vaccine demonstrated safety and immunogenicity in adult volunteers. A Phase II study in two- to five-year-old children is planned. To produce a bivalent vaccine, nontoxic shiga toxin I purified from mutant *E. coli* O157 will be conjugated with O-specific PS (O-PS). The major reservoir of *E. coli* O157 is cattle. A LPS-protein conjugate was shown to be immunogenic in mice and cattle, and a challenge study is planned to demonstrate the clearance of the carriage state in cattle.

*Vibrio cholerae* O1 and O139 are the major serotypes in cholera infections. Conjugates synthesized with CPS of O139 and O-PS of LPS elicited vibriocidal antibodies in mice. Clinical trials of these conjugates are planned.

## SELECTED PUBLICATIONS

1. Lin FY, Ho VA, Khiem HB, Trach DD, Bay PV, Thanh TC, Kossaczka Z, Bryla DA, Shiloach J, Robbins JB, Schneerson R, Szu SC. The efficacy of a Salmonella typhi Vi conjugate vaccine in two-to-five-year-old children. N Engl J Med. 2001;344:1263-1269.
2. Schwebach JR, Casadevall A, Schneerson R, Dai Z, Wang X, Robbins JB, Glatman-Freedman A. Expression of a Mycobacterium tuberculosis arabinomannan antigen in vitro and in vivo. Infect Immun. 2001;69:5671-5678.
3. Schwebach JR, Glatman-Freedman A, Gunther-Cummins L, Dai Z, Robbins JB, Schneerson R, Casadevall A. Glucan is a component of the Mycobacterium tuberculosis surface that is expressed in vitro and in vivo. Infect Immun. 2002;70:2566-2575.

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# HUMAN IMMUNE RESPONSE TO POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES

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Surface polysaccharides (PS) of pathogenic bacteria, including capsusalar PSs (CPS) or lipopolysaccharides (LPS), serve as both essential virulence factors and protective antigens. The age-related and T cell–independent immunogenicity of CPS limit their use as vaccines, especially in infants and young children. LPS are too toxic to be administered. Accordingly, their O-specific PSs (O-SP), which shares the virulence-promoting and protectiveness properties of CPS, must be purified. However, O-SP are too small to be immunogenic (haptens). Covalent binding of CPS or of O-SP to medically useful proteins to form conjugates both increases their immunogenicity and confers T-cell dependence on these saccharides. Part of the work of this laboratory is directed at making synthetic vaccines to improve their immunogenicity.

## Conjugate Vaccines against Shigellosis

The O-SP of *Shigella sonnei* and of *Shigella flexneri* 2a were bound to bacterial toxoids. In adult army recruits and then in four- to seven-year-old children, both conjugates proved to be safe and induced statistically significant and long-lived rises in IgG antibody levels against the homologous LPS. Similar though lesser increases in IgM and IgA anti–LPS levels were also induced. Re-injection of *S. flexneri* 2a conjugate induced a booster response in the recruits as well as in the four- to seven-year-old children. A Phase III trial showed that one injection of *S. sonnei* O-SP, bound to a nontoxic recombinant *Pseudomonas aeruginosa* exoprotein A (rEPA), protected army recruits against outbreaks of this pathogen. Importantly, we noted a statistically significant correlation between the levels of serum IgG anti–LPS and the efficacy of the conjugate. We developed two methods that increased the immunogenicity of the Shigella conjugates in mice: another carrier protein, a genetically inactivated *Corynebacterium diphtheriae* toxin (CRM9), was a superior carrier for *S. sonnei* O-SP, and treatment of rEPA with succinic anhydride, a nontoxic mild akylating agent that converts amino groups of proteins to carboxyls, increased the immunogenicity of *S. flexneri* 2a O-SP. A Phase I study of these Shigella conjugates in adults confirmed their safety and immunogenicity; the improved immunogenicity was less marked than in mice. A Phase II study in one- to four-year-old children showed an improved immunogenicity of the new *S. flexneri* 2a conjugate but less immunogenicity of the *S. sonnei* conjugate. A Phase III study of the modified *S. flexneri* 2a and the original *S. sonnei* conjugates is in preparation. In collaboration with the Lanzhou Vaccine Institute and Provincial Medical Center in Henan, China, a clinical trial of the two conjugates is in the planning stage.

### Conjugate Vaccine against *Staphylococcus aureus*

*Schneerson, Robbins; in collaboration with Shinefeld, Black, Fattom, Horwith, Fuller, Ordonez, Rasgon, Yoh, Law, Johnson, Alcom, Muenz, Naso*

*Staphylococcus aureus,* an opportunistic pathogen, is a major cause of bacteremia and its complications in patients with decreased resistance. The pathogen is becoming increasingly resistant to last-resort antibiotics such as methicillin and vancomycin. Its CPS is essential for pathogenesis of and immunity to *S. aureus*. In a randomized, double-blinded, placebo-controlled study conducted in about 1,800 hemodialysis patients, we found that our conjugate vaccine consisting of the CPS linked to recombinant *Pseudomonas aerugi-nosa* nontoxic exotoxin A significantly decreased the number of *S. aureus* infections. Given that such patients are least likely to respond to immunoprophylaxis, the vaccine's efficacy is likely to be higher in other patients at risk for *S. aureus* infection.

### Recombinant Vaccine against *Bacillus anthracis*

*Schneerson; in collaboration with Leppla, Ramirez, Shiloach*

The virulence of *Bacillus anthracis* is mediated by a capsule and an exotoxin consisting of protective antigen (PA), lethal factor, and edema factor. Antibodies against PA are thought to be protective. The currently available vaccine, produced by treating *B. anthracis* culture supernatants with formalin, has a relatively high rate of side effects and has to be administered several times to elicit protective immunity. Moreover, because it consists of a mixture of undefined components, it is difficult to standardize. To overcome these problems, we developed culture conditions and purification procedures to produce sufficient quantities of a genetically modified PA (rPA) to test in mice. The resultant rPA vaccine elicited high levels of IgG anti-PA antibodies with neutralizing activities in mice.

### Use of Cross-Reacting Antigens to Boost Immungenicity

To determine if concurrent administration of a cross-reacting and homologous CPS has an advantage over the use of the homologous CPS alone, we used conventional methods to isolate the cell wall PS of *Bacillus pumilus*, SH18, which has been reported to cross-react with the CPS of *Haemophilus influenzae* type b (HIb). We then investigated its structure by using gel chromatography–mass spectrometry and showed that it contains glycerol, ribitol, and 2-acetamido-2-deoxyglucose, in a molar ratio of 0.2:1.0:0.2 and 17 percent phosphate. The CPS cross-reacted with anti–*Staphilococcus epidermidis* as well as with the anti-HIb. Methods to prepare a conjugate of this PS are under investigation.

### Other Vaccines

*Neisseria meningitidis* group A causes endemic and epidemic meningitis, notably in the meningitis belt of Africa. An effective and available CPS vaccine is underused. To improve the vaccine's immunogenicity further, as in the case of other CPS, we are investigating methods of binding it to a carrier protein. We developed a double mutant of *Bordetella pertussis*, producing a genetically inactivated toxin deficient in FHA synthesis. Effort is currently directed toward increasing production of this *B. pertussis* strain as a more easily purified pertussis toxin for a monocomponent vaccine and as a carrier protein for pneumococcal type 14 CPS. *Clostridium difficile* is a major cause of hospital-acquired diarrhea following antibiotic usage:

the diarrhea caused by the pathogen is mediated by two exotoxins, A and B. Toxin A, considered to be the major toxin, can, in extreme cases, cause pseudomembranous colitis. A genetically derived toxin mutant (rARU) both induces antitoxin and protects animals from infection with *C. difficile*. The succinylation of rARU improved its solubility and did not detectably affect its antigenicity. Techniques to prepare a mutant toxin A for clinical use have been worked out. We conjugated three PSs of varying composition from pneumococcus type 14, *Escherichia coli* K1, and *S. flexneri* 2a to succinylated rARU. The resultant conjugates induced high levels of both antiPS and antitoxin. Preparation of toxin A conjugates for clinical evaluation is under way. *Borrelia burgdorferi*, a spirochete transmitted though the bite of infected *Ixodes* ticks, is the etiologic agent of Lyme disease. A protein vaccine against it is available but is not effective under the age of 12 years. Lipopolysaccharide (LPS) has been described in other spirochetes, but its presence in *B. burgdorferi* has been debated. So far, we have not been able to confirm its presence. The search for LPS revealed a unique glycolipid consisting of glycerol and galactose as the carbohydrate moiety. Evidence suggests that this glycolipid is surface-exposed. Injected in complete Freund's adjuvant, it induced specific antibodies.

### Synthetic Vaccines against *Shigella dysenteriae* Type 1 and *Shigella sonnei*

*Ekborg, Sampath-Kumar, Nelson, Pozsgay, Robbins, Schneerson; in collaboration with Liptak*

*Shigella dysenteriae* type 1 and *Shigella sonnei* are Gram-negative human pathogens that cause endemic and epidemic dysentery throughout the world. Despite the pathogens' discovery many years ago, no licensed vaccines exist to protect against the bacteria, which have developed resistance to most available antibiotics. Our approach to vaccine development is based on the demonstration that serum antibodies against the O-SPs of Gram-negative bacteria are important for host protection. While O-SPs are nonimmungenic, presumably because of their low molecular weight, covalent conjugates of an immunogenic protein and the O-SP of *S. dysenteriae* type 1 and *S. sonnei* have been shown to elicit significant anti–O-SP antibody levels of the IgG isotype, which may be boosted by repeated injections. We surmised that an improved vaccine might be constructed from chemically defined oligosaccharide fragments of O-SPs, which are devoid of biological contamination, carry a uniform molecular weight, and can be characterized by physicochemical methods.

We have chemically synthesized oligosaccharide fragments of the native O-SP of *S. dysenteriae* type 1 up to a tetracosasaccharide consisting of 24 monosaccharide residues. These constructs are attached to a spacer that makes bioconjugation possible through the termini of the spacer moieties (i.e., at a single site). We have been developing a new technique for chemical synthesis of extended oligosaccharides that circumvents the difficulties associated with the conventional, liquid-phase coupling of oligosaccharide building blocks and avoids the problems inherent in the solid-phase approaches. In brief, our method employs lipophilic protecting groups in one of the blocks while using conventional protecting groups in the others. The presence of the lipophilic groups in the targeted intermediate but not in the side-products allows the isolation of the products by the solid-phase extraction technique that uses recyclable chromatographic materials and environmentally friendly alcoholic solvents. To improve the efficiency of oligosaccharide synthesis by facilitating product isolation, we have designed and synthesized several new lipophilic protecting groups, including 4-dodecyloxybenzyl, 4-dodecylbenzyl, and 4-octadecylbenzyl moieties. The utility of these groups has been documented. We are currently using our new technology for the synthesis of the oligosaccharides related to the O-SP of *S. dysenteriae* type 1 under clean laboratory conditions for Phase I clinical trials. So far, we have demonstrated that the immunogenicity of the synthetic saccharide–human serum albumin conjugates depends on the saccharide chain length and the saccharide density on the protein, the latter in a nonlinear fashion.

Current work is directed at determining the role, if any, of the monosaccharide terminating the oligosaccharide chain. To this end, we have synthesized oligosaccharide fragments of the O-SP that differ in the terminal unit, i.e., in the repeating unit frame. We designed experiments aimed at making a PS mimic that might be more immunogenic when conjugated to proteins than the saccharides synthesized so far. In brief, we designed linearly arranged "clusters" of the synthetic oligosaccharides that are interconnected by spacers through predefined sites. We demonstrated the feasibility of this approach by interconnecting six monosaccharide units. Next, we extended this approach to a synthetic octasaccharide. So far, we have succeeded in interconnecting three such units, leading to a polymer containing 24 monosaccharide residues. At present, we are trying to synthesize even longer constructs as protein conjugates for immunological experiments.

We developed a new technique for reagentless attachment of oligosaccharides to proteins. In short, the technique is based on the Diels-Alder cycloaddition between an activated double bond and a conjugated diene. The activated double bond component was introduced in the protein by using the commercial reagent 3-sulfosuccinimidyl 4-maleimidobutyrate. The carbohydrate sector was equipped with various linkers containing a 1,3 conjugated diene system. When solutions of the functionalized components were combined, conjugation took place at room temperature. pH has a marked effect on the efficiency of the coupling: lower pH (e.g., pH 5.7) favors conjugation compared with higher pH (e.g., 9.5). The conjugation was as effective in water as in pH 5.7 buffer. On the other hand, the structure of the diene system has little effect on the conjugation reaction. An advantage of this method over existing methods is that the unconjugated saccharide can be recovered with its linker for reuse in a subsequent conjugation experiment.

## Synthetic Vaccines against Group A *Neisseria meningitidis*

*Berkin, Coxon, Yergey, Pozsgay, Robbins, Schneerson*

Group A *Neisseria meningitidis* causes endemic and epidemic meningococcal meningitis worldwide. Although a vaccine containing the purified CPS of the bacterium has been available for years, it is not sufficiently immunogenic in infants, who are at highest risk. Our program to develop a more efficient vaccine is based on the assumption that protein conjugates of relatively short fragments of the CPS (i.e., which can be made available by controlled chemical synthesis) of Group A *N. meningitidis* will be more immunogenic in infants than the currently available vaccine. The CPS of Group A *N. meningitidis* consists of alpha-linked N-acetyl-mannosaminyl residues that are interconnected by an anomerically located phosphodiester linkage. The native PS is nonstoichiometrically O-acetylated at the O-3 position. In our first approach to studying the requirements of synthetic oligosaccharides related to this polymer, we targeted the synthesis of non–O-acetylated congeners. We have designed a route to the mannosaminyl H-phosphonate moiety. Iterative condensation of the unit allowed the synthesis of a dimer and trimer of the PS's repeating unit in bioconjugatable form. We also synthesized the mannosaminyl spacer and its phosphate ester. Covalent attachment of the synthetic subunits to human serum albumin using our conjugation technology afforded glycoconjugates containing up to 30 saccharide chains per molecule of HSA. The antigenicity of the conjugates was demonstrated by the precipitin formation with the serum of a horse that was immunized with formalin-killed Group A *N. meningitidis*. The experiments showed that the serum recognizes as small a component of the PS as a monosaccharide. The formation of precipitation indicates that the presence of O-acetyl groups in the polymer is not essential for antigenicity. Current work is directed toward the synthesis of higher oligomers with and without O-acetyl groups for evaluation of their immunogenicities.

## SELECTED PUBLICATIONS

1. Passwell J, Harlev E, Ashkenazi S, Chu C, Miron D, Ramon R, Farzan N, Shiloach J, Bryla D, Majadly F, Roberson R, Robbins J, Schneerson R. Safety and immunogenicity of improved shigella O-specific polysaccharide-protein conjugate vaccines in adults in Israel. Infect Immun. 2001;69:11351-11357.
2. Ramirez DH, Leppla, SH, Schneerson R, Shiloach J. Production, recovery and immunogenicity of the protective antigen antigen from a recombinant strain of *Bacillus anthracis*. J Indust Microbiol Biotechnol. 2002;28:232-238.
3. Robbins JB, Schneerson R, Trollfors B. Pertussis in developed countries. Lancet. 2002;360:657-658.
4. Shinefield H, Black S, Fattom A, Horwith G, Rasgon S, Ordonez J, Yeoh H, Law D, Robbins JB, Schneerson R, Muenz L, Fuller S, Johnson J, Fireman B, Alcorn H, Naso R. Use of a Staphylococcus aureus conjugate vaccine in patients receiving hemodialysis. N Engl J Med. 2002;346:491-496.
5. Taranger J, Trollfors B, Bergfors E, Knutsson N, Lagergard T, Schneerson R, Robbins JB. Immunologic and epidemiologic experience of vaccination with a monocomponent pertussis toxoid vaccine. Pediatrics. 2001;108:E115.
6. Taranger J, Trollfors B, Bergfors E, Knutsson N, Sundh V, Lagergard T, Lind-Brandberg L, Zackrisson G, White J, Cicirello H, Fusco J, Robbins JB. Mass vaccination of children with pertussis toxoid—decreased incidence in both vaccinated and nonvaccinated persons. Clin Infect Dis. 2001;33:1004-1010.
7. Trollfors B, Lagergard T, Taranger J, Bergfors E, Schneerson R, Robbins JB. Serum immunoglobulin G antibody responses to Bordetella pertussis lipooligosaccharide and B. parapertussis lipopolysaccharide in children with pertussis and parapertussis. Clin Diagn Lab Immunol. 2001;8:1015-1017.

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# DEVELOPMENTAL REGULATION OF NEURONAL AND MUSCLE PLASTICITY

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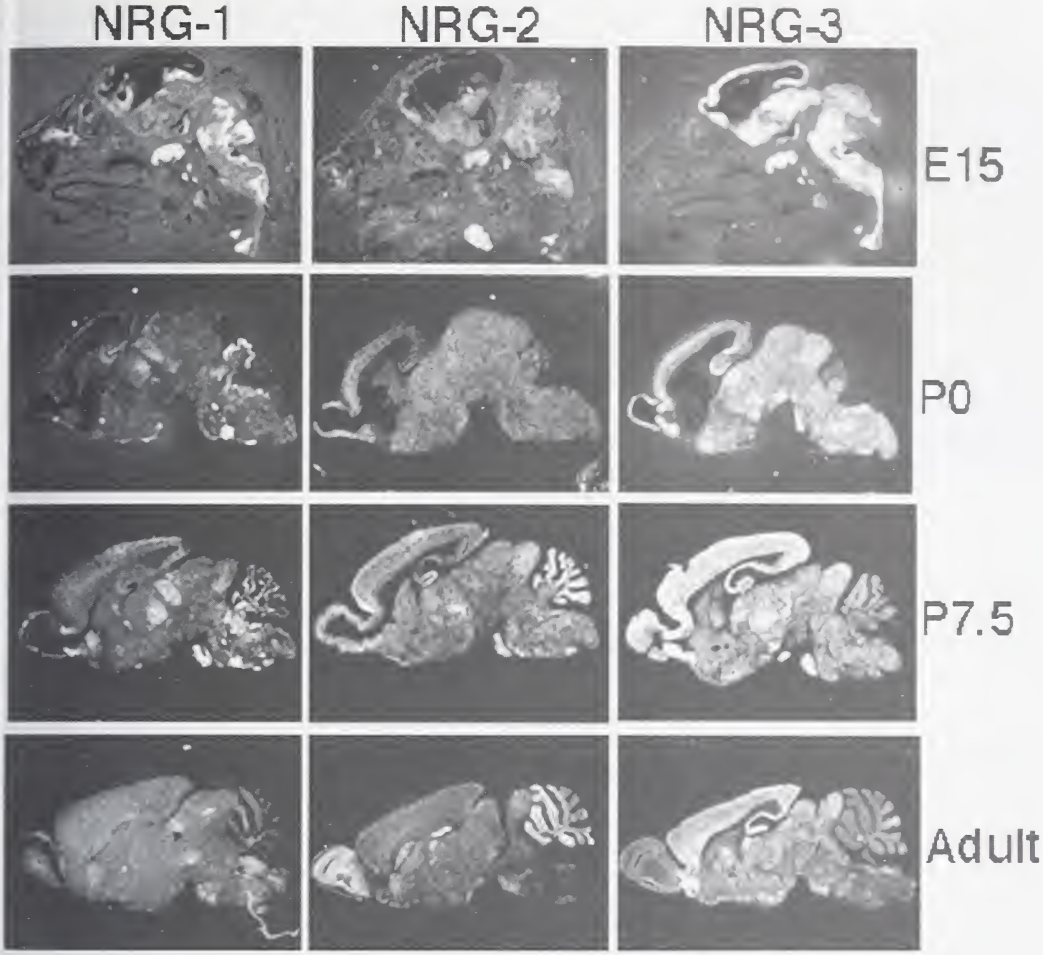
The general interest of the laboratory lies in understanding the molecular mechanisms that regulate neuronal and muscle plasticity during development. Until recently, a commonly held view in neurobiology was that neural inputs (particularly neuronal activity) are instructive to the functional properties of postsynaptic targets. Recently, a different picture has begun to emerge. Early in development and independently of innervation, lineage plays a fundamental role in the pre-patterning of targets. Later, during the maturation of neural connections, activity can modify the plastic properties of postsynaptic targets. Moreover, the view that electrical activity exerts its effects predominantly by regulating the levels of intracellular calcium is undergoing revision because of the realization that the action of growth/differentiation factors on synaptic function is activity-dependent and can occur in the time scale of minutes. We are using two experimental models to investigate how neural factors and activity regulate neuronal and muscle plasticity during development. In the first project, we are investigating how neuregulins acutely regulate synaptic transmission at interneuronal synapses. In the second, we are studying how lineage and motoneuron activity contribute to the emergence and plasticity of different muscle types.

## Neuregulin Effects on Synaptic Plasticity: Possible Role in Schizophrenia

*Buonanno*

Neuregulins (NRG 1-3) are growth/differentiation factors that signal by means of a family of receptor tyrosine kinases known as ErbB 1-4. Present knowledge of NRGs mostly originates from studies on NRG-1. The pro-NRG-1 is synthesized as a transmembrane precursor in the soma, then transported down axons, and proteolytically released in its active form in an activity-dependent fashion. Earlier work by our group and others showed that long-term exposure (more than two days) of neurons to NRG-1 elicits changes in the composition of neurotransmitter receptors for glutamate (NMDA subtype), GABA, and acetylcholine by selectively regulating the expression of distinct receptor subunits. We found that co-activation of both glutamate and ErbB receptors is necessary for NRG-1 to induce NMDA receptor expression, suggesting a cross-talk between these signaling pathways. The subsequent demonstration that ErbB4 and NMDA receptors co-localize at glutamatergic synapses with PSD-95, a PDZ protein coupling postsynaptic receptors to signaling complexes, led us to hypothesize that the NRG/ErbB signaling pathway may acutely modify synaptic properties (Garcia *et al.*, 2000).



Expression of NRG-1, NRG-2, and NRG-3 mRNAs in the developing central nervous system. Sections from E15, P0, P7.5, and adult mice were hybridized with specific $P^{33}$-labeled cRNA probes for NRGs 1–3. The regional and developmental profiles of each NRG differ, suggesting that they may have different biological functions.

### NRG and ErbB Receptor Expression in the Developing Central Nervous System

*Longart, Liu, Vasudevan, Karavanova, Buonanno; in collaboration with Carroll*

We investigated the regional and temporal expression of NRGs and their receptors because such information is essential for understanding how the NRG/ErbB pathway contributes to neuronal function. We used multiple approaches in our studies, including *in situ* hybridization, Western blots, and immunofluorescence histochemistry. The patterns of NRG 1-3 mRNA expression differ markedly during development (Fig. 20). In general, NRG-1 expression is highest early in development and becomes restricted postnatally. NRG-2 mRNA levels in embryonic and newborn mouse brains are low compared with those of NRG-3. Seven days after birth, the highest levels of NRG-2 are found in the dentate gyrus, olfactory bulb, and cerebellum. In contrast, NRG-3 is the most highly expressed NRG in the brain and shows the least regional or developmental regulation. To study the cellular and subcellular distribution of these proteins in brain, we generated and characterized antibodies against NRG-2 and NRG-3. Preliminary studies indicate that NRG-2 and NRG-3 accumulate in neurons, although expression in glia cannot be ruled out. Interestingly, the subcellular distributions of NRG 1-3 differ, suggesting that NRGs may perform distinct functions during neuronal development and maturation.

Although ErbB receptors are critical for neural development, their regional distributions during development and in the adult were unknown. ErbB receptors are differentially expressed in neurons and glia. ErbB2 is expressed in most cells, ErbB3 is predominantly found in glia and subpopulations of neurons, and ErbB4 is mostly restricted to neurons and oligodendrocytes. The punctate labeling with ErbB2 and ErbB4 antibodies is highest in synapse-rich regions, which is consistent with the previous finding that ErbB receptors accumulate at postsynaptic densities (Garcia *et al.*, 2000). ErbB4 receptors are mostly restricted to the dendrites and cell bodies of GABAergic neurons, where they co-localize at excitatory synapses with NMDA receptors and PSD-95. The existence of complexes of these proteins at postsynaptic densities suggests that the NRG/ErbB pathway may function to regulate synaptic function acutely.

### NRG-1 Acutely Modifies Synaptic Transmission in Cultured Hippocampal Neurons

*Liu, Longart, Buonanno; in collaboration with Vicini*

To test the aforementioned hypothesis, we analyzed the acute affects of NRG on synaptic transmission. A brief application of 5nM NRG1-$\beta$1 (about two minutes), which causes the phosphorylation of ErbB2 and ErbB4, induces a persistent and significant increase in the firing patterns of cultured hippocampal neurons. These effects are specific because they are not observed with the NRG-$\alpha$2 splice variant that differs slightly with NRG1-$\beta$1 and is a weaker activator of ErbB receptors. The findings suggest that NRGs may play important roles in synchronizing synaptic or network activity of hippocampal circuitry. Experiments are in progress to understand the underlying mechanisms regulating synaptic plasticity in response to NRG in dissociated hippocampal fresh slices and cultures.

The implications of these findings for basic and clinical science may be extremely important in light of a recent study associating NRG-1 mutations with schizophrenia in Icelandic families. Moreover, mutant mice with decreased levels of NRG-1 and ErbB receptors have fewer NMDA receptors and manifest behavioral deficits reported to be consistent with schizophrenia. Pharmacological agents used to treat schizophrenia reverse these behavioral changes in mutant mice.

### Contribution of Developmental History and Neural Activity to the Fiber-Type Specificity of Troponin I Genes

*Buonanno*

The developmental and neuronal regulation of skeletal muscle fiber types provides an excellent model in which to study how patterned activity regulates plasticity of postsynaptic targets. Our long-term objectives are to identify transcription factors that both regulate the emergence of slow- and fast-twitch fibers during development and intraconvert their contractile properties in response to specific patterns of motoneuron activity. The troponin I slow (TnIs) and fast (TnIf) genes serve as our experimental paradigm because their expression is fiber-type–specific and regulated by selective patterns of electrical impulses that mimic motoneuron activity. We have determined that the General Transcription Factor 3 (GTF3), expressed in numerous tissues including muscle and brain, contributes to the establishment of fiber types during perinatal development (Calvo *et al.*, 2001).

### Transcription Factor GTF3 Contributes to the Slow Muscle Program

*Vullhorst, Karavanova, Buonanno*

The TnIs is activated during terminal myogenic differentiation in all skeletal muscles regardless of their future fiber type and is later confined to prospective slow fibers during fetal development. The SURE element (for slow upstream regulatory enhancer), which confers slow fiber specificity to TnIs expression, requires interactions between multiple transcription factors. The *bicoid*-like motif (BLM) in SURE is bound by GTF3 and is required for slow fiber-specific expression. GTF3 is expressed at its highest levels during fetal development in

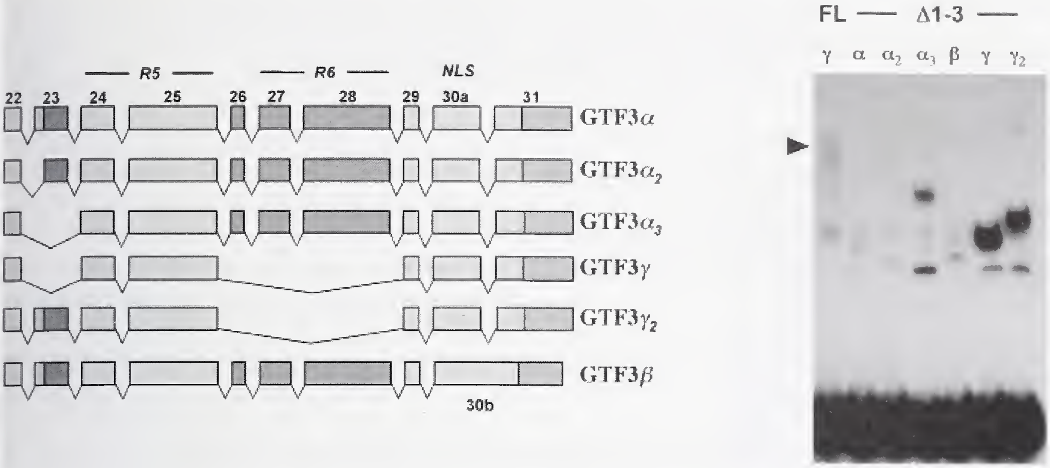


**FIGURE 21**

GTF3 splice variants bind to the SURE BLM with different avidities. *Left:* Partial 3' exon-intron structure of mouse GTF3 splice variants isolated from skeletal muscle. Dark gray boxes represent alternatively spliced exons, dark portions of 3' exons 30a and 30b are noncoding. *Right*: Electrophoretic mobility shift assays of full-length ('FL') mouse GTF3γ and aminoterminally truncated (Δ1-3') mutant versions of GTF3α, β, and γ isoforms. Arrowhead indicates specific shift obtained with full-length GTF3γ.

numerous tissues, and its expression in muscles is repressed after birth. Experiments in adult regenerating muscles (which re-express GTF3) transfected with GTF3 by using electroporation, as well as analysis of GTF3 mutant mice, support the role of GTF3 in regulating the slow-twitch muscle program.

### Biochemical Characterization of GTF3 and Its Novel Splice Variants

*Vullhorst, Buonanno*

To map the GTF3 DNA binding domain, we generated a series of human GTF3 constructs harboring different truncations. In TFII-I, the paralog of GTF3, a basic motif that maps between the reiterated helix-loop-helix (HLH) domains R1 and R2 is necessary for DNA binding. In contrast, analysis of numerous GTF3 deletion constructs identified the HLH domain 4 (R4) as necessary and sufficient to mediate DNA binding and showed that sequences in the N-terminal region interfere with binding. The mouse GTF3 gene gives rise to α, β, and γ splice variants that differ in sequences carboxyterminal to the DNA-binding domain R4. To determine which GTF3 splice variants are expressed in skeletal muscle, we employed RT-PCR. We identified three novel splice variants (called GTF3 α2, α3, and γ2 for their exonic structure), in addition to the known GTF3 α1 and γ1 isoforms, in all muscle and non-muscle tissues examined. Using EMSAs, we compared the interactions between the different GTF3 splice variants with the BLM. As was the case for the human GTF3, the full-length mouse proteins interacted poorly with the BLM, but N-terminally truncated versions of GTF3 α3, γ1, and γ2 avidly bound to the probe. These experiments suggest that muscle gene expression may be differently regulated by distinct GTF3 isotypes.

Next, we investigated if an aminoterminal leucine zipper–like (LZ) motif in GTF3 is required for homomeric or heteromeric interactions with TFII-I. We followed the interactions of GTF3 with itself or with TFII-I by using full-length and truncated GTF3 proteins on co-immunoprecipitation assays (co-IP) and Western blots. The experiments demonstrated that GTF3 polypeptides interact with each other, but not with TFII-I, via the LZ domain. We confirmed the significance of the interactions in myoblasts by using immunofluorescence cytochemistry. A GTF3 mutant protein (GTF3.D3-6) lacking a *bona fide* nuclear localization signal located near the carboxyterminus remains cytosolic unless co-expressed with full-length GTF3. In agreement with the co-IP experiments, GTF3.D3-6 fails to localize to the nucleus when co-expressed with a full-length GTF3 lacking only the LZ zipper (GTF3. DLZ). In conclusion, GTF3 can form complexes via the LZ motif with itself, but not with TFII-I, indicating that both transcription factors act independently and possess distinct DNA binding properties.

### Possible Implications of GTF3 and GTF2i in Williams Syndrome

*Vullhorst, Karavanova, Buonanno*

The genes encoding GTF3 and its related homolog GTF2i are deleted in individuals with Williams syndrome (WS). The biochemical characterization of GTF3 (and GTF2i) and the study of its function *in vivo* could provide important information about the molecular basis for WS. Patients suffering from WS have distinctive physical, cognitive, and behavioral abnormalities that include impaired spatial cognitive skills and myopathies. WS is a rare, sporadic disorder resulting from the loss (haplotype) of approximately 20 genes (including the *gtf3* and *gtf2i*) located in about 2.0 Mb of chromosome 7q11.23. Recent studies strongly implicate GTF3 and TFII-I as candidate proteins that contribute to the deficiencies observed in WS patients. Our studies using ectopically transfected GTF3 constructs in adult muscles and GTF3 knock-out mice strongly support a role for this factor in regulating muscle contractile properties, which could be related to myopathies observed in WS. The observation that GTF2-I and GTF3 are mostly expressed in developing musculature and neurons raises the possibility that reduction of these factors during embryogenesis could affect the expression of target genes later in development.

## SELECTED PUBLICATIONS

1. Buonanno A, Fischbach G. Neuregulin and ErbB receptor signalling in the nervous system. Curr Opin Neurobiol. 2001;11:287-296.
2. Calvo S, Vullhorst D, Venapally P, Karavanova I, Cheng J, Buonanno A. Molecular dissection of DNA sequences and factors involved in slow muscle-specific transcription. Mol Cell Biol. 2001;21:8490-8503.
3. Desai A, Turetsky D, Vasudevan K, Buonanno A. Analysis of the NMDA receptor 2A subtype gene in transgenic mice and in transfected cortical neurons. J. Biol. Chem. 2002; 277:46374-46384.
4. Garcia R, Vasudevan K, Buonanno A. The neuregulin receptor ErbB-4 interacts with the PDZ domain protein at neuronal synapses. Proc Natl Acad Sci USA. 2000;97:3596-3601.
5. Gerecke KM, Wyss JM, Karavanova I, Buonanno A, Carroll SL. ErbB transmembrane tyrosine kinase receptors are differentially expressed in adult rodent central nervous system. J Comp Neurol. 2001;433:86-100.
6. Longart M, Buonanno A. Neuregulins: a family of factors with critical functions during nervous system development and in the cellular transformation and differentiation. Rev Neurol. 2002;34:91-97.
7. Villegas R, Villegas G, Hernandez M, Longart M, Maqueira B, Buonanno A, Garcia R, Castillo C. Neuregulin found in cultured sciatic nerve conditioned medium causes neuronal differentiation of PC12 cells. Brain Res. 2001;852:305-318.

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# TETANUS AND BOTULINUM NEUROTOXINS AND NEURONAL CELL BIOLOGY

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Using biochemical and morphologic techniques, our research focuses on synaptic function in primary neuronal cell cultures. Studies utilize the clostridial neurotoxins (tetanus and seven serotypes of botulinum) whose catalytic domains are zinc endopeptidases that block vesicular neurotransmitter release by cleaving specific proteins implicated in synaptic vesicle fusion with the presynaptic membrane. Consequently, the toxins have become valuable tools for understanding neurotransmitter release, membrane trafficking, and protein sorting, transport, and targeting. The identity of toxin receptor(s), organelles involved in toxin uptake, the mechanism of toxin translocation into the neuronal cytoplasm, intracellular trafficking of the toxins, and toxin effect on synaptic vesicle trafficking remain subjects of active research. Intoxication *in vivo* with botulinum neurotoxin causes a fatal flaccid paralysis of voluntary muscle. Given their paralytic effect, the toxins are important therapeutic agents for a number of neurologic disorders characterized by excessive muscle contraction, including cerebral palsy. Botulinum poisoning remains a public health problem, and the toxin is considered a potential agent of bioterrorism; a more complete understanding of its intracellular functioning will aid in designing an appropriate therapy.

## Synaptic Vesicle Recycling

*Parfitt, Behar*

We reported previously that the action of botulinum neurotoxin (BoNT) serotype A on cultured spinal cord neurons was unique among the *C. botulinum* toxin serotypes in that it appeared to block synaptic vesicle exocytosis while allowing some endocytosis to continue (Neale *et al.*, J Cell Biol. 1999;147:1249). In an effort to examine this phenomenon with improved temporal resolution and sensitivity, we have monitored synaptic vesicle endocytosis and exocytosis by the quantitative kinetic measurement of synaptic staining and destaining with fluorescent FM dyes. Both BoNT A–blocked and untreated cultures are stained with FM2-10 during five minutes of potassium depolarization in the presence of dye. In control cultures, essentially all the FM dye is lost on subsequent depolarization. The initial fast phase of destaining fits a single exponential; a second, slower process sometimes manifests after one to two minutes. BoNT A–treated cultures, which take up about 50 percent less FM dye than controls, lose only about 50 percent on destaining and show markedly different destaining kinetics. The rate of the initial fast phase is reduced compared with the controls and lasts for a shorter time. The rate of the second process is greatly reduced. In control cultures in the presence of a very low extracellular calcium concentration, destaining kinetics are analogous to those seen with normal calcium in BoNT A–treated cultures.

The pool of synaptic vesicles is believed to comprise three subgroups: a reserve pool that does not normally participate in synaptic transmission; a recycling pool (RP) that is fully stainable after prolonged exposure to FM dyes; and a readily releasable pool (RRP) containing vesicles that are rapidly exocytosed at the start of high-frequency stimulation and that have been identified morphologically (by others) as those vesicles docked at the active zone.

Based on our current findings, we propose that part of the lethal effect of BoNT A intoxication may be attributable to the severe retardation of the rate of replenishment of vesicles in the RRP rather than solely to a direct inhibition of vesicle exocytosis. Similarly, low calcium may allow fusion of readily releasable vesicles but not support movement of vesicles from the recycling to the readily releasable pool. We will test our hypothesis by examining the distribution of labeled vesicles in the electron microscope after brief periods of depolarization.

### Uptake and Translocation of Botulinum Neurotoxin

*Keller, Behar*

BoNT serotypes A and E cleave the same synaptic protein, although BoNT A is more potent and remains active for longer than BoNT E (Keller *et al.*, FEBS Lett. 1999;456:137; Keller *et al.*, 2001). Potassium stimulation greatly enhances the uptake of BoNT into spinal cord neurons in cell culture, implicating recycling synaptic vesicles as the uptake compartment. Short exposure to toxin in depolarizing medium produces a saturable uptake. Movement of toxin across the vesicle membrane into the neuronal cytosol requires low intravesicular pH. Bafilomycin A1 inhibits the vacuolar ATPase, preventing vesicle acidification. The drug was used to trap toxin within vesicles in order to study the synchronized translocation of toxin across the vesicle membrane. Toxin translocation occurs about 25 minutes after uptake and is complete within 90 minutes. Bafilomycin is less effective at blocking the translocation of BoNT E than of BoNT A, indicating that BoNT E is able to cross the membrane in the presence of a more shallow pH gradient. This difference may reflect a fundamental difference in intracellular toxin trafficking, perhaps relevant to the long duration of action of BoNT A. The data indicate that the potency of BoNT A is related to an aspect of toxin action other than the efficiency of translocation.

## SELECTED PUBLICATIONS

1. Keller JE, Neale EA. The role of SNAP-25 in the potency of botulinum neurotoxin type A. J Biol Chem. 2001;276:13476-13482.
2. Neale EA. Botulinum neurotoxin A and synaptic vesicle trafficking. In: Brin M, Hallett M, Jankovic J, eds. Proceedings of the International Conference 1999: Scientific and Therapeutic Aspects of Botulinum and Tetanus Toxins. New York: Lippincott, Williams and Wilkins, 2002;61-74.

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# MECHANISMS OF NEURODEVELOPMENT IN HEALTH AND DISEASE

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We seek to determine the epigenetic mechanisms through which environmental factors influence development of the nervous system. In particular, we are interested in both the coupling between electrical and synaptic activity in neural systems and the growth, differentiation, and maturation of neurons and their connections in synaptic circuits. Elimination of redundant, non-functional synapses is a major process involved in nervous system development, and we have demonstrated some of the molecular and cell-biologic mechanisms responsible for activity-dependent synapse elimination in an *in vitro* model system. We seek to extend our studies to some neurodevelopmental disorders such as autism.

## Activity-Dependent Synapse Loss and Stabilization at the Neuromuscular Junction

*Nelson, Jia, Li, Viloria, Yang, Lanuza; in collaboration with Fields*

The Hebbian, activity-dependent modulation of synapse efficacy, in which activated pathways are strengthened and inactive ones weakened, depends on an integrated pattern of activation of protein kinases, such that appropriate phosphorylation reactions occur that differentially affect the stability of activated and non-activated synapses and their associated receptors. One step in the activation of the kinases, in particular protein kinase C (PKC), involves the action of the serine protease, thrombin, and its associated receptor, the protease-activated receptor or PAR. Thrombin's action can be reproduced by a peptide, the thrombin receptor–activating peptide (TRAP), which can activate the PAR. Collaborative experiments with Dr. Lanuza and her group in Spain have shown that the thrombin receptor (ThR) is localized at the neuromuscular junction *in vivo* and that application of the TRAP increases the rate of synapse elimination that occurs naturally in the Lanuza group's system. Thus, the molecular apparatus for thrombin's participation in the synapse modification process is present and functional at the neuromuscular junction. The natural elimination process can be divided into three stages: an early stage in which significant loss of polyneuronal innervation occurs with relatively little change in the distribution of post-synaptic receptors, an intermediate stage in which both axonal loss and receptor changes are prominent, and a final stage in which the innervation has become primarily mono-neuronal, although substantial changes in the receptors occur. It is during the early/intermediate stage that pharmacological and genetic prevention of PKC action blocks synapse elimination. Genetically modified (PKC theta knock-out) animals show a delay of synapse loss developmentally, but eventually the muscle innervation becomes completely mononeuronal. The use of PKC blockers *in vivo* over the same period also completely prevented synapse elimination. We have investigated what aspect of the neural activation process might be involved in the kinase-mediated change in synapse effectiveness. Carbachol can be used to provide cholinergic stimulation of skeletal muscle, and we have shown that such treatment of cultured skeletal myotubes results in activation and membrane translocation of PKC theta.

A variety of experiments have implicated PKA as playing a crucial role in the stabilization of activated synapses. In view of the redundant mechanisms underlying activity-dependent synapse elimination implied by the studies of PKC block or knock-out, we were interested in alternative messengers or mediators of the elimination/stabilization. In collaboration with Douglas Fields, we have examined the possibility that ATP might be involved in the process. For this purpose, we have used apyrase, an enzyme that breaks down ATP, and suramin, a blocker of the P3 purinergic receptor that mediates some of the effects of ATP. In the presence of these agents, stimulation produces a significant decrement in synapse strength (stimulation of control, untreated preparations do not produce a decrement of the stimulated inputs). The depression lasts for over an hour after the drugs are washed out and stimulation of the preparation terminated. Both nerve and muscle in our preparations release large amounts of ATP, with the release modulated by activation of the nerve or muscle. Thus, it seems possible that ATP may play a role in the stabilization of activated synapses, possibly through activation of a purinergic receptor.

Activation of the PI3 kinase by Wortmannin does not affect synapse stability in either resting or stimulated synapses, leading to the conclusion that phosphorylations in general are not involved in synapse modulation. It seemed worthwhile to explore further the possibility that kinases other than PKA and PKC were involved in activity-dependent synapse modulation. The ras-mitogen–activated protein kinase (MAPK) and cAMP/PKA-mediated pathways play important roles in neuronal plasticity. We have used different blockers of the MAPK to test for its involvement in synapse modification in our system. Two such blockers are PD98059 and UO128. Neither of these produces synapse loss in the absence of synapse activation, but, in conjunction with stimulation, both do produce loss. In the presence of synapse stimulation, the inactive analog of UO128, UO124, produces no synapse loss. The results suggest that MAPK may be involved in the Hebbian synapse modulation that occurs in our model system.

### Molecular Bases for Neurodevelopmental Disorders

*Nelson, Kohler, Satyanarayana, Krek-Larner;* in collaboration with Grether*

We have used a variety of immunoaffinity analytical methods to determine the levels of several neuro-active molecules in blood samples from controls and children with different neurodevelopmental disorders. Through collaboration with members of the California Department of Health Services, we have access to blood specimens that were drawn at birth from a large sample of children and archived with subsequent diagnosis of some of the children as having autism or other developmental disorders. In very-low-birth-weight children with and without diagnoses of infection or cerebral palsy, we found no correlation of clinical condition with levels of a number of cytokines. Our finding stands in contrast to earlier results showing significant differences in cytokine levels that correlated with clinical condition in term infants. We have measured the neonatal blood levels of the neurotrophins NT-3, NT-4, and Brain Derived Neurotrophic Factor (BDNF) and of several cytokines in controls and in children subsequently shown to be autistic. We observed no significant differences between cases and controls for IL-1 or IL-8, but the level of BDNF was about 25 percent lower in samples for autistic cases than in normals. We obtained our result with the double antibody sandwich immunoaffinity method used with the bead flow–based technique of the Luminex system. Given that our result did not show the increase in BDNF (as well as some other analytes) found earlier with the single antibody recycling immunoaffinity chromatographic method, we are attempting to develop a single antibody version of the Luminex method to determine if methodological issues explain the discrepancy.

## SELECTED PUBLICATIONS

1. Kim S, Bondeva T, Nelson PG. Activation of protein kinase C isozymes in primary mouse myotubes by carbachol. Brain Res Dev Brain Res. 2002;137:13-21.
2. Lanuza MA, Garcia N, Santafe M, Alonso J, Gonzalez C, Nelson PG, Tomas J. Pre and postsynaptic motor endplate maturation during neonatal synapse elimination depends on protein kinase C. J Neurosci Res. 2002;67:607-617.
3. Li M-X, Jia M, Yang L-X, Dunlap V, Nelson PG. Pre and post-synaptic mechanisms in Hebbian activity-dependent synapse modification. J Neurobiol. 2002;52:241-250.
4. Nelson KB, Nelson PG. Size of head and brain in autism: clue to underlying biologic mechanism? In: Bauman M, Kemper T, eds. Neurobiology of autism. Baltimore: Johns Hopkins University Press;in press.

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# NEUROPEPTIDE-MEDIATED REGULATION OF DEVELOPMENT

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We investigate vasoactive intestinal peptide (VIP) as a regulator of brain development and neuroprotection. VIP can influence many important processes that are central to development, including the survival of nerve cells, neurite extension, excitatory synaptogenesis, and embryonic growth. Many of the neurotrophic and growth-stimulating actions of VIP are mediated indirectly through secreted, glia-derived substances. Much of our effort is devoted to identifying substances whose release is triggered by VIP and its mechanism of action. After identifying the VIP-related, neuroprotective substances, the continuing goal is to develop therapeutic agents that prevent neurodegenerative disease.

### Activity-Dependent Neurotrophic Factor

*Hill, Li, Zhao, Hauser, McCune, Spong, Brenneman; in collaboration with Gozes, Jaffee, Yergey*

Previous studies have indicated that one of the protective proteins released by VIP is a femtomolar-acting protein: activity-dependent neurotrophic factor (ADNF). One of our continuing priorities is to characterize ADNF at the protein and genetic levels and to develop therapeutic agents based on peptides derived from ADNF. Recent evidence indicates that ADNF is a complex of proteins characterized by three peaks of biological activity that greatly differ in potency to prevent apoptotic death in cell cultures derived from the central nervous system. During the last year, the fundamental nature of the ADNF-related protective activity has become apparent: multiple proteases are involved. Through the use of specific inhibitors of protease action, all the survival-promoting activity can be inhibited. Furthermore, the protease activity of one of the ADNF components has been demonstrated in a cell-free system by using a fluorescent peptide substrate. The protease activity detected with this methodology corresponded very closely to the potency of the survival-promoting activity. The data strongly support the hypothesis that ADNF is a multi-protease complex that can increase the survival of developing neurons exposed to toxic agents that produce apoptotic death. Thus, studies on the protease activity of component II of the ADNF complex emphasize the proof of principle; however, all the neurotrophic activities of the ADNF components may reside in their proteolytic actions.

### Neuroprotective Peptides

*Spong, Goodwin, Hauser, Poggi, Brenneman; in collaboration with Gozes*

Previous studies have shown that short peptides derived from ADNF and a pharmacologically related protein (activity-dependent neuroprotective protein; ADNP) exhibit neuroprotection at femtomolar concentrations. The protective action of these peptides is observed in cultures treated with clinically relevant toxins, including glutamate, beta amyloid peptide, iron, hydrogen peroxide, and gp120, the HIV envelope protein. Recently, 11 new peptides derived from ADNF have been discovered, although a number of the structures show homology to known proteases. This is an interesting corollary in that the biological activity ADNF complex is now clearly linked to protease activity. All 11 new peptides were tested for intrinsic survival-promoting activity by utilizing a cell culture model of tetrodotoxin-induced neuronal cell death. The experiments indicated that four of the 11 peptides showed potent survival-promoting activity. Both neuronal cell counts and the carboxyfluorescene diacetate methodology used to assess neuronal survival confirmed complex dose/response relationships. The new peptides provide further basis for the exploration of these structures as lead compounds for therapeutic intervention in the treatment of neurodegenerative disease.

### VIP and the Neural Tube

*Hill, McCune, Sahir, Brenneman*

VIP is an important regulator of embryonic growth and development during the early post-implantation period of development, with VIP receptors localized to the neural tube. We have used explanted neural tubes to perform studies of VIP-mediated regulation of gene expression and neurotrophin release. With this model system, we have shown that nerve growth factor (NGF) is one of the factors regulated by VIP in neural tube preparation explants from the early post-implantation mouse embryo. Although a small amount of NGF was found in the fully processed form, VIP stimulation releases NGF primarily in the form of a prohormone from the embryonic mouse neural tube preparation. VIP induced significantly more immunoreactive NGF in both conditioned medium and within the neural tube preparation itself than was found in untreated neural tube preparations and in preparations treated with equal concentrations of the highly homologous neuropeptide, PACAP.

The VIP concentrations used were within the peptide's biologically active range in central nervous system tissues, and immunoreactive NGF was released at concentrations that elicit biological actions. However, the concentrations of immunoreactive NGF in the conditioned medium represent NGF diluted into the incubation medium and suggest that, within the local environment of neural tube tissues, VIP stimulates the release of NGF prohormone at much greater concentrations.

The model system has revealed a functional relationship between VIP and NGF, and the findings support the hypothesis that VIP mediates development through its action as a secretagog. The study links, for the first time, the actions of VIP with releasable NGF prohormone in neural tissue. The results of this work imply that drugs mimicking the neurotrophic action of VIP in the central nervous system could elicit protective and repair modalities of NGF and thereby are of therapeutic value.

## SELECTED PUBLICATIONS

1. Ashur-Fabian O, Giladi E, Furman S, Steingart RA, Wollman Y, Fridkin M, Brenneman DE, Gozes I. Vasoactive intestinal peptide and related molecules induce nitrite accumulation in the extracellular milieu of rat cerebral cortical cultures. Neurosci Lett. 2001;307:167-170.
2. Brenneman DE, Hauser JM, Spong CY, Phillips, TM. Chemokine release is associated with the protective action of PACAP-38 against HIV envelope protein neurotoxicity. Neuropeptides. 2002;36:271-280.
3. Gelber E, Granoth R, Fridkin M, Dreznik Z, Brenneman DE, Moody TW, Gozes I. A lipophilic vasoactive intestinal peptide analog enhances the antiproliferative effect of chemotherapeutic agents on cancer cell lines. Cancer. 2001;92:2172-2180.
4. Hill JM, Mehnert J, McCune SK, Brenneman DE. Vasoactive intestinal peptide regulation of nerve growth factor in the embryonic mouse. Peptides. 2002;23:1803-1808.
5. Leker RR, Teichner A, Grigoriadis N, Ovadia H, Brenneman DE, Fridkin M, Giladi E, Romano J, Gozes I. NAP, a femtomolar-acting peptides, protects the brain against ischemic injury by reducing apoptotic death. Stroke. 2002;33:1085-1092.

6. Moody TW, Leyton J, Chan D, Brenneman DE, Fridkin M, Gelber E, Levy A, Gozes I. VIP receptor antagonist and chemotherapeutic drugs inhibit the growth of breast cancer cells. Breast Cancer Res Treat. 2001;1911:1-10.
7. Poggi SH, Vink J, Goodwin K, Hill JM, Brenneman DE, Pinhasov A, Gozes I, Spong CY. Differential expression of embryonic and maternal activity dependent neuroprotective protein during development. Amer J Obstet Gyn. 2002;187:973-976.
8. Romano J, Beni-Adani L, Nissenbaum OL, Brenneman DE, Shohami E, Gozes I. A single administration of the peptide NAP induces long term protective changes against the consequences of head injury. J Mol Neurosci. 2002;18:37-45.
9. Sharma A, Walters J, Gozes Y, Fridkin M, Brenneman DE, Gozes I, Moody T. A vasoactive intestinal peptide antagonist inhibits the growth of glioblastoma cells. J Mol Neurosci. 2001;17:331-339.
10. Spong CY, Auth J, Vink J, Goodwin K, Abebe DT, Hill JM, Brenneman DE. Vasoactive intestinal peptide mRNA and immunoreactivity are decreased in fetal alcohol syndrome model. Reg Pept. 2002;108:143-147.

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# BIOLOGICAL RHYTHMS: THE PINEAL GLAND

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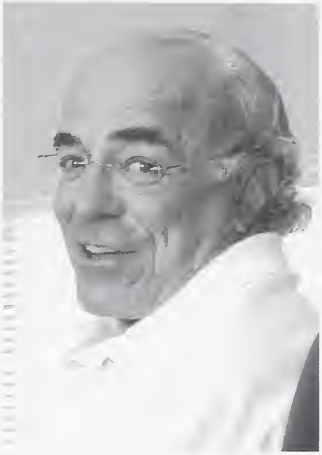

Research efforts leading to a better understanding of biological rhythms will have a broad influence on all aspects of health and the treatment of disease. Twenty-four hour and seasonal rhythms are fundamental features of all forms of life, reflecting the adaptation of life to dominant features of the earth's environment. Our daily 24-hour routines, including the sleep/wake cycle, are controlled by a complex neural system that has an endogenous master circadian oscillator and an important output signal, melatonin, the hormone of time. Light acts on this system to entrain the endogenous clock with environmental lighting. Circulating melatonin is produced in the pineal gland and is always elevated at night. Our primary objective is to advance our knowledge of vertebrate chronobiology and neuroendocrinology. Our efforts focus on understanding the regulation of the daily rhythm in melatonin production, which plays an essential role in mental health and in timing seasonal and circadian biology. Melatonin is used in humans with the intention of improving the entrainment of the biological clock in the blind and in individuals suffering from "jet lag" resulting from shift work and travel.

### Post-Translational Regulation of the Melatonin Rhythm Enzyme: Arylalkylamine N-Acetyltransferase

*Klein, Coon, Gaildrat, Ganguly, Morin, Kim, Weller; in collaboration with Aitken, Boutin, Cole, Zhen, Dyda, Hickman, Obsil, Falcón, Ho, Chik, Iuvone, Jaffe, Korf, Schomerus, Malpaux, Chemineau, Markey, Kowalak, Ron, Toyama, Dawid, Young*

The enzyme that drives the day/night rhythm in melatonin production is arylalkylamine N-acetyltransferase (AANAT). Many regulatory mechanisms converge on this enzyme. Our studies have provided evidence for at least three post-translational mechanisms involved in cyclic AMP regulation of AANAT activity: phosphorylation-dependent prevention of proteolytic destruction, phosphorylation-dependent allosteric effects, and uncharacterized events that activate the protein without altering the phosphorylation state of AANAT. A highly conserved mechanism involved in regulating AANAT activity is prevention of proteolysis. Studies of several species, representing three vertebrate classes, have pointed to a close association between changes in AANAT activity and AANAT protein levels. We prepared antisera against AANAT from ungulates, primates, rodents, chicken, and fish. In all cases, the antisera demonstrated a close association between large changes in AANAT protein and activity, including the large *in vivo* day/night changes and the rapid reductions that occur when lights are turned on in the middle of the night. We established that the rapid decrease in AANAT protein is due to proteolysis by the proteasome, an extremely rapid and irreversible mechanism that turns off melatonin production, a mechanism that is regulated by cyclic AMP.

AANAT is typically phosphorylated in the cell. A hunt for phosphorylation-dependent AANAT binding identified 14-3-3 proteins. The binding to AANAT is triggered by phosphorylation of at least one and probably both PKA sites on the enzyme. The N-terminal PKA site is located in a sequence that becomes a consensus 14-3-3 binding motif upon phosphorylation (RRHTPLAN → RRHpTPLAN). Crystallographic analysis of the AANAT/14-3-3 complex revealed that the PKA/14-3-3 motif forms multiple contacts with residues in the amphipathic binding groove of 14-3-3, which allowed us to propose a simple model of the complex. Other studies revealed that the binding could be disrupted by using a 16-residue peptide containing the PKA/14-3-3 binding motif. Although the 14-3-3/AANAT complex has been studied only in the ovine pineal gland, it is probable that, given the highly conserved nature of the PKA/14-3-3 binding motif of AANAT and the ubiquitous presence and highly conserved structure of 14-3-3 proteins, AANAT exists in this form in all vertebrates. In accordance with evidence that 14-3-3 prevents dephosphorylation by a phosphatase and clipping of the N-terminal tail by a protease, the mechanism through which proteolysis of AANAT is regulated appears to involve shielding by 14-3-3.

Phosphorylation-dependent binding of AANAT to 14-3-3 protein not only protects against proteolysis but also activates AANAT by increasing the affinity of AANAT for serotonin. Analysis of the crystal structures of AANAT, obtained with and without a bisubstrate inhibitor, indicates that a region of AANAT known as Loop 1 is involved in this allosteric effect. 14-3-3 stabilizes Loop 1 in a configuration that favors catalysis in general and strongly influences serotonin binding, causing a 10-fold increase in the affinity of the enzyme for serotonin. We created cell lines expressing *Aanat* and used them to study the effect of the level and phosphorylation state of the enzyme on its function. Intracellular acetylation was activated by elevation of cyclic AMP levels without changing total AANAT protein, pointing to the effects of cyclic AMP on other factors involved in acetylation, such as the availability of AcCoA.

### Pineal/Retinal Tissue-Specific Gene Expression

*Klein, Coon, Shi, Weller; in collaboration with Baler, Carter, Humphries, Falcón, Gothilf, Ron, Toyama, Dawid*

The pineal expresses an unusual set of genes, some of which are expressed in only one other tissue: the retina. To determine the molecular events involved in directing tissue-specific expression of these genes, we used regions of the rat and zebra fish *Aanat* promoter, first, to insert genes of interest into the pineal gland, which has allowed us to characterize promoters that drive expression in the pineal gland in both animals, and, second, to study the effects of overexpression of genes of interest. To probe the function of Fra-2, which is induced in coordination with AANAT in the rodent pineal gland, we developed a construct that knocks down expression of the transcription factor Fra-2 the rat. However, the knockdown does not alter induction of AANAT. Work in zebrafish has led to the development of a line of fish that expresses the green fluorescent protein reporter gene in the pineal gland under control of 3' and 5' elements of the AANAT gene flanking the open reading frame. The line has potential use in analysis of the factors that regulate the developmental expression of the AANAT gene.

### Evolution of AANAT

*Klein, Coon, Shi; in collaboration with Falcón, Gothilf, Koonin*

The discovery of two AANAT enzymes in fish triggered interest in the possibility that two genes exist in other vertebrates. We have so far failed to find evidence of a second AANAT in any vertebrate other than fish. Duplication of the fish genome appears to have occurred following the emergence of fish from the evolutionary line leading to higher vertebrates and to have allowed distinctly different enzymes to evolve in fish, with different kinetic characteristics, and, in some species, remarkable differences in tissue distribution. In trout, one *Aanat* gene is expressed in the pineal *(Aanat-2)* and the other in the retina *(Aanat-1)*. Regulatory differences have also evolved; in the trout pineal gland, AANAT is high at night and suppressed by light, as seen in all vertebrates, but light increases AANAT in the trout retina. These differences may have evolved to meet the different pressures on melatonin function in the pineal gland (hormonal signal of time) as compared with that in the retina (paracrine control of photosensitivity).

### Transcriptional Control of *Aanat*

*Klein, Coon, Weller; in collaboration with Baler, Blackshaw, Carter, Humphries, Chong, Iuvone*

Although post-translational mechanisms play an important role in regulating AANAT activity and protein levels, the abundance of mRNA encoding the enzyme is controlled in some vertebrates. For example, day/night changes in AANAT mRNA are large in birds and rodents, whereas day/night AANAT mRNA levels are similar in trout, sheep, cows, and primates. Thus, we have studied transcriptional control of AANAT by Fra-2, a transcription factor that has been thought to control expression of AANAT in the rat. The AANAT promoter has been used as a vehicle to confer pineal- and retinal-specific expression of genes. A genetically engineered rat has been produced in which Fra-2 protein is knocked down. However, suppression of Fra-2 does not alter AANAT expression, although it was found to influence expression of the orphan receptor NGFI-B (Nur77/TR3), a member of a nuclear hormone receptor subfamily that includes the related factors Nurr1 and NOR-1. This finding has opened new opportunities to examine the role that NGFI-B plays in biology and the role that Fra-2 plays in regulating this gene. Research on chicken *Aanat* has identified an E-box element, which mediates circadian expression of some clock-controlled genes such as the AANAT gene. Other studies establish that the circadian clock in the chicken pineal gland is directly linked to *Aanat* by transcription factors that are part of the molecular machinery that makes up the biological circadian clock, providing a direct link from the clock to chicken *Aanat.*

### Primate Retinal Melatonin Synthesis

*Klein, Coon, Weller; in collaboration with Young*

We maintain an interest in the regulation of melatonin in the human pineal gland and have used the rhesus pineal gland and retina as models of the human tissues, making it was possible to study elements of the serotonin-to-melatonin pathway. As is the case in all vertebrates, the activity and protein levels of AANAT in the pineal and retina were elevated at night. However, as discussed above, there were no day/night differences in AANAT mRNA, indicating that AANAT activity is regulated in primates at a post-translational site, probably by the binding of 14-3-3 proteins. A striking finding was that HIOMT mRNA was low or undetectable in the retina, consistent with previous studies on post mortem human retinae and the bovine retina. Such low levels in the primate retina suggest that AANAT may not be dedicated to melatonin production and might have a somewhat different role in the retina, such as in the synthesis of N-acetylserotonin, which may act in place of melatonin as a ligand for melatonin receptors. Alternatively, N-acetylserotonin may have an anti-oxidant role. Another possible role for AANAT is that it acts to prevent the accumulation of toxic derivatives of aromatic amines.

### Pineal Protein Hunt

*Klein, Coon, Gaildrat, Ganguly, Morin, Kim, Weller; in collaboration with Baler, Blackshaw, Carter, Humphries, Malpaux, Chemineau, Markey, Kowalak*

With the goal of defining a map of signal transduction and metabolic networks that describe pineal function, we are constructing a comprehensive list of proteins that are of special importance to pineal function. More specifically, we are working toward identifying and characterizing proteins that bind to AANAT in order to develop a better understanding of AANAT's regulation. Fabrice Morin undertook protein-protein interaction screens of an ovine pineal cDNA library in yeast by using AANAT as the bait (yeast two-hybrid screen). The work revealed an interaction between AANAT and an enzyme that makes AcCoA, ATP-dependent citrate lyase (ACL). ACL was highly expressed in the pineal gland compared with other tissues and exhibited a day/night difference in phosphorylation state. Preliminary studies suggest that ACL and AANAT associate in the ovine pineal gland and that AcCoA generated by the ACL is preferentially channeled to

AANAT. Two collaborations used gene chips/microarrays as a first step toward identifying pineal proteins that exhibit large day/night changes that are photically regulated and that are highly expressed in the pineal gland. With commercially available chips, we found a rhythm in the transcription factor Id1. We hope to characterize fully the rhythm in NGFI-B protein and, using microarray technology, to analyze the expression of genes in animals in which expression of Fra-2 was knocked down selectively in the pineal gland and retina. We are also using custom chips containing probes for genes expressed by retinal cones, brain genes, and ESTs from pineal and retinal libraries. With these, we have been able to detect daily rhythms in mRNA encoding the S-adenosylmethionine–synthesizing enzyme, methionine adenosyl transferase (MAT), and adrenergic regulation of MAT mRNA, with high levels occurring at night, which is when the demand for S-adenosylmethionine is highest.

## SELECTED PUBLICATIONS

1. Coon SL, del Olmo E, Young WS, Klein DC. Melatonin synthesis enzymes in a non-human primate pineal gland: focus on Rhesus arylalkylamine N-acetyltransferase (EC 2.3.1.87). J Clin Endocrinol Metab. 2002;87:4699-4706.
2. Coon SL, Weller JL, Korf HW, Namboodiri MAA, Rollag M, Klein DC. cAMP regulation of arylalkylamine *N*-acetyltransferase (AANAT, EC 2.3.1.87): a new cell line (1E7) provides evidence of intracellular AANAT activation. J Biol Chem. 2001;276:24097-24107.
3. Falcón J, Gallarneau K, Coon SL, Weller JL, Ron B, Chen G, Klein DC. Regulation of arylalkylamine *N*-aceytltransferase-2 (AANAT2, 2.3.1.87) in the fish pineal organ: evidence for a role of proteasomal proteosomal proteolysis. Endocrinology. 2001;142:1804-1813.
4. Ganguly S, Coon SL, Klein DC. Control of melatonin synthesis in the mammalian pineal gland. Cell Tissue Res. 2002;309:127-137.
5. Ganguly S, Gastel J, Weller JL, Schwartz C, Jaffe H, Namboodiri MAA, Coon SL, Hickman AB, Rollag M, Obsi T, Beauverger P, Ferry G, Boutin JA, Klein DC. Role of a pineal cAMP-operated arylalkylamine *N*-acetyltransferase/14-3-3 binding switch in melatonin synthesis. Proc Natl Acad Sci USA. 2001;98:8083-8088.
6. Ganguly S, Mummaneni P, Steinbach PJ, Klein DC, Coon SL. Characterization of the *Saccharomyces cerevisiae* homolog of the melatonin rhythm enzyme arylalkylamine *N*-acetyltransferase (AANAT, EC 2.3.1.87). J Biol Chem. 2001;276:47239-47247.
7. Gothilf Y, Toyama R, Coon SL, Du SJ, Dawid IB, Klein DC. Pineal-specific expression of green fluorescent protein under the control of the serotonin-N-acetyltransferase gene regulatory regions in transgenic zebrafish. Dev Dynam. 2002;225:241-249.
8. Humphries A, Klein DC, Baler R, Carter DA. cDNA array analysis of pineal gene expression reveals circadian rhythmicity of the dominant negative helix-loop-helix protein-encoding gene, Id-1. J Neuroendocrinol. 2002;14:101-108.
9. Iuvone PM, Brown AD, Haque R, Weller JL, Zawilska JB, Chaurasia SS, Ma MH, Klein DC. Retinal melatonin production: role of proteasomal proteolysis in circadian and photic control of arylalkylamine *N*-acetyltransferase. Invest Opthalmol Vis Sci. 2002;43:564-572.
10. Klein DC, Ganguly S, Coon SL, Weller JL, Obsil T, Hickman A, Dyda F. 14-3-3 proteins and photoneuroendocrine transduction: role in controlling the daily rhythm in melatonin. Biochem Soc Trans. 2002;30:365-372.
11. Obsil T, Ghirlando R, Klein DC, Ganguly S, Dyda F. Crystal structure of the 14-3-3ζ:serotonin N-acetyltransferase complex: a role for scaffolding in enzyme regulation. Cell 2001;105:257-267.
12. Smith M, Burke Z, Humphries A, Wells T, Klein DC, Carter D, Baler R. Tissue specific transgenic knock-down of *fos* related antigen-2 (Fra-2) expression mediated by dominant negative Fra-2. Mol Cell Biol. 2001;21:3704-3713.

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# PROTEIN TRAFFICKING AND SECRETORY GRANULE BIOGENESIS IN NEUROENDOCRINE CELLS

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We study the cell biology of endocrine and neuroendocrine cells. Our work focuses on the mechanisms of biosynthesis and intracellular trafficking of peptide hormones and neuropeptides and their processing enzymes. Our studies have led to the discovery of novel molecular mechanisms of protein trafficking to the regulated secretory pathway and of recycling of sorting receptors as well as to the understanding of diseases related to defects in hormone targeting. We have also identified a master on/off switch that regulates dense-core granule biogenesis in neuro-endocrine and endocrine cells.

### Mechanism of Sorting Pro-Neuropeptides to the Regulated Secretory Pathway

*Cawley, Zhang, Dhanvantari, Rodriguez-David, Arnaoutova, Loh*

The intracellular sorting of pro-neuropeptides to the regulated secretory pathway (RSP) is essential for processing, storage, and release of active hormones in the neuroendocrine cell. We thus investigated the sorting of pro-opiomelanocortin (POMC, pro-ACTH/endorphin) and pro-enkephalin (proEnk) to the RSP. We showed that, as a concentration step, these pro-proteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum to the trans-Golgi network (TGN); there, they are sorted into dense-core granules of the RSP for processing and secretion. Site-directed mutagenesis studies identified a consensus sorting motif consisting of two acidic residues 12–15Å apart and exposed on the surface of these molecules and two hydrophobic residues 5–7Å away from the acidic residues, which are necessary for sorting to the RSP. We identified an RSP sorting receptor that was specific for the sorting signal of POMC and proEnk as membrane carboxipeptidase E (CPE). The two acidic residues in the prohormone sorting motif specifically interact with two basic residues, R255 and K260, of the CPE sorting receptor to effect sorting to the RSP.

We showed that CPE is a transmembrane protein that is anchored in the TGN via its C-terminal amphipathic tail to unique cholesterol-glycosphingolipid–rich microdomains known as rafts. Removal of cholesterol from secretory granule membranes resulted in the inability of CPE to bind to cargo; by treating cells with lovastatin, cholesterol depletion resulted in lack of sorting of CPE and POMC to the RSP. Thus, membrane association is essential for the prohormone sorting receptor function of CPE at the TGN. Transfection of CPE bearing R255A and K260A mutations into a CPE null clone of Neuro2a cells and transfection of a dominant negative CPE construct into AtT20 cells caused missorting of POMC to the constitutive pathway, indicating that the basic residues in the sorting domain of CPE interact with the acidic residues in the POMC sorting signal *in vivo* to effect sorting to the RSP. Using a mouse model that synthesizes a mutant CPE, which is degraded in the pituitary, we were able to show missorting of endogenous POMC in these neuroendocrine cells. Our studies provide evidence for a sorting signal/receptor-mediated mechanism for targeting prohormones to the RSP in neuroendocrine cells.

### Sorting of Proinsulin Mutants in Hyperproinsulinemia Patients

*Dhanvantari, Zhang, Loh; in collaboration with Mackin, Morris*

Investigations into the sorting of proinsulin in pancreatic β-cells has identified an RSP sorting signal in proinsulin similar to POMC. In monomeric proinsulin, the sorting signal motif consists of residues E13 and L17 located on the B chain and L16 and E17 located on the A chain. In hexameric proinsulin, residue E13 on the B chain is buried, and two residues in the A chain from two adjacent proinsulin dimers in the hexamer contribute a motif. Depletion of CPE in these pancreatic cells with siRNA and the use of a dominant negative CPE construct demonstrated that CPE acts as a sorting receptor for proinsulin in β-cells. Furthermore, the binding of proinsulin and insulin to membrane CPE has pH optima of pH 6.5 and 5.5, respectively. We have proposed a "sorting by retention" model for proinsulin sorting in β-cells, which differs from the "sorting by entry" model for POMC sorting in neuroendocrine cells. In β-cells, proinsulin binds to CPE at the TGN pH of 6.5 and is sorted into immature secretory granules (ISG). However, other non–RSP proteins such as the lysosomal enzymes also enter the ISG in β-cells. Proinsulin is then released from CPE in the ISG and processed to insulin. Insulin then rebinds CPE at the more acidic pH of the ISG and is retained while other non-RSP proteins are removed from the ISG by a constitutive-like pathway (Fig. 22). To understand the molecular basis of hyperproinsulinemia, we investigated the intracellular sorting of genetically mutated proinsulins found in such diabetes patients who, by definition, have abnormally high levels of plasma proinsulin. One form of mutant proinsulin found in the patients, HisB10Asp, which is unable to hexamerize but forms dimers, missorted to the constitutive pathway and was secreted in an unregulated manner when transfected into a cell line. Molecular modeling of the dimer of this mutant proinsulin predicted that the molecular distance between the two acidic residues of the RSP sorting signal motif would be too large to allow interaction with the basic residues in the binding site of the sorting receptor, CPE. Indeed, *in vitro* binding studies showed that the mutant did not bind to CPE, resulting in its inability to be sorted to the RSP for processing to insulin and secretion in a secretogogue-dependent manner. Other hyperproinsulinemia proinsulin mutants R65P and R65L were also secreted constitutively and were not stored. Binding studies showed that mutant R65P and R65L proinsulins bound poorly to CPE, accounting for the lack of sorting and retention in the ISG (Fig. 22). The high levels of secreted mutant proinsulins in the plasma of the hyperproinsulinemia patients are therefore attributable to defects in sorting of these molecules as a consequence of their genetic structural alterations.

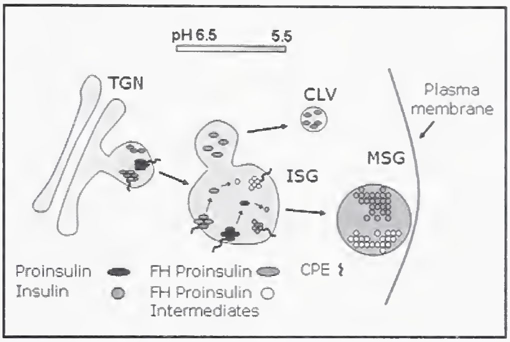

FIGURE 22

Schematic diagram showing a model of proinsulin trafficking to the regulated secretory pathway in β-cells.

### Sorting and Recycling of the Sorting Receptor CPE

*Zhang, Dhanvantari, Arnoutova, Loh; in collaboration with Donaldson, Jackson*

Our studies showed that CPE, a transmembrane protein, is sorted into granules of the RSP in neuroendocrine cells by a mechanism involving insertion of its C-terminal domain into lipid rafts at the TGN. Upon stimulation and exocytosis, membrane CPE is localized on the plasma membrane and remains associated with rafts. It is subsequently recycled back to the TGN by a novel internalization mechanism. The internalization of CPE from the plasma into early endosomes requires not only functional ARF6, a GTPase, but also physical interaction of ARF6 with the six-amino-acid cytoplasmic tail of CPE.

### Regulation of Secretory Granule Biogenesis

*Kim, Loh; in collaboration with Eiden, Cheng*

Formation of large dense-core granules (LDCG) at the TGN is essential for regulated secretion of hormones and neuropeptides from neuroendocrine cells. Our recent studies uncovered a master on/off switch, chromogranin A (CgA), that controls the formation of LDCG in neuroendocrine cells. Using antisense technology, we observed that depletion of CgA in rat PC12 cells resulted in the loss of LDCG and regulated secretion, and led to degradation of the granule proteins CgB and synapto-tagmin. Over-expression of bovine CgA in the same cells rescued the wild-type phenotype. In a mutant endocrine cell line, 6T3, which lacks CgA, LDCGs, and regulated hormone secretion, transfection of CgA restored the wild-type phenotype. Recently, we used microarrays to compare gene expression in 6T3 cells lacking LDCGs with 6T3 cells stably transfected with CgA. We found that the aquaporin 1 (AQP1, a water channel) and the granuphilin genes were highly up-regulated in 6T3 cells expressing CgA, indicating a new role of CgA at the transcriptional level. While it is known that granulphilin is present in LDCGs and is important in exocytosis, the subcellular localization and role of AQP1 in endocrine cells is unclear. We have now shown that 6T3 cells transfected with CgA and pituitary and chromaffin cell secretory granules contain high levels of AQP1 protein. Thus, AQP1 may be important in regulating secretory granule volume during granule maturation and exocytosis.

## SELECTED PUBLICATIONS

1. Cawley NX, Chino M, Maldonado A, Rodriguez YM, Loh YP, Ellman J. Synthesis and characterization of the first potent inhibitor of yapsin 1: implications for the study of yapsin-like enzymes. J Biol Chem. 2002; (e-pub ahead of print).
2. Cawley NX, Loh YP. Yapsin 1. In: Barrett AJ, Woessner JF, eds. Handbook of proteolytic enzymes, 2d ed. London: Academic Press, 2002;in press.
3. Dhanvantari S, Arnaoutova I, Snell CR, Steinbach PJ, Hammond K, Caputo GA, London E, Loh YP. Carboxypeptidase E, a prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion. Biochemistry. 2002;41:52-60.
4. Dhanvantari S, Cawley NX, Loh YP. Prohormone convertases. In: Martini L, ed. The encyclopedia of endocrinology and endocrine diseases. 2002;in press.
5. Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Chromogranin A, an “on/off” switch controlling dense-core granule biogenesis. Cell. 2001;106:499-509.
6. Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Large dense-core secretory granule biogenesis is under the control of chromogranin A in neuroendocrine cells. Ann NY Acad Sci. 2002;971:323-331.
7. Loh YP, Cawley NX, Yapsin A. In: Barrett AJ, Woessner JF, eds. Handbook of proteolytic enzymes, 2d ed. London: Academic Press, 2002;in press.
8. Loh YP, Maldonado A, Zhang CF, Tam WH, Cawley NX. Mechanism of sorting pro-opiomelanocortin and pro-enkephalin to the regulated secretory pathway of neuroendocrine cells. Ann NY Acad Sci. 2002;971:416-425.
9. Volpi S, Rabadan-Diehl C, Cawley NX, Aguilera G. Transcriptional regulation of the pituitary vasopressinV1b receptor involves a GABA binding protein. J Biol Chem. 2002;277:27829-27838.

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# TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL MECHANISMS IN NUTRIENT REGULATION OF GENE EXPRESSION

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We are studying how the yeast *Saccharomyces cerevisiae* regulates the expression of its genome according to nutrient availability. In response to starvation for an amino acid, purine, or glucose, a global regulatory mechanism known as general amino acid control mediates the transcriptional induction of about one-fifth of the genome, including most genes encoding amino acid or purine biosynthetic enzymes. Synthesis of the transcriptional activator *GCN4* is stimulated under starvation conditions by a translational control mechanism involving short open reading frames (uORFs) in the *GCN4* mRNA leader and the general translation initiation factors eIF2 and eIF2B. The guanine nucleotide exchange factor eIF2, which recycles inactive eIF2-GDP to active eIF2-GTP after each round of initiation, forms a ternary complex (TC) with initiator $\text{tRNA}_i^{\text{Met}}$ and GTP and binds to the 40S ribosomal subunit. The induction of *GCN4* translation depends on phosphorylation of the α-subunit of eIF2 on serine-51 by the protein kinase GCN2, converting eIF2 from a substrate to inhibitor of eIF2B. Phosphorylation of eIF2 by GCN2 reduces the rate of general protein synthesis but simultaneously induces translation of *GCN4* mRNA. In this way, the cell can decrease the demand for amino acids while increasing its capacity for the acids' *de novo* production.

### Importance of Direct eIF2-eIF3 Contact in the Multifactor Complex for Translation Initiation *in Vivo*

*Valasek, Nielsen, Hinnebusch*

Assembly of the 80S translation initiation complex is a multiple-step process involving a large number of soluble eukaryotic initiation factors (eIFs). According to current models, the TC binds to the 40S ribosome with the help of eIFs 1, 1A, and 3. The 43S preinitiation complex thus formed interacts with mRNA in a manner stimulated by eIF4F and poly(A)-binding protein, and the resulting 48S complex scans the mRNA until the Met-tRNA$_i^{\text{Met}}$ base-pairs with the AUG start codon. On AUG recognition, the eIF5 stimulates GTP hydrolysis by eIF2, the eIFs are ejected, and the 60S subunit joins with the 40S-Met-tRNA$_i^{\text{Met}}$-mRNA complex in a reaction stimulated by eIF5B.

We showed previously that yeast eIF3 contains only five subunits (TIF32, PRT1, NIP1, TIF35, and TIF34). We also demonstrated that eIF5, the GTPase-activating protein (GAP) for the TC, and eIF1, a factor regulating AUG start codon selection, both copurify with eIF3 (Phan *et al.*, 2001; Asano *et al.*, 2001). Through protein interaction assays with recombinant subunits, we produced a subunit interaction map for eIF3 in which PRT1 is the scaffold of the complex. The two smallest subunits (TIF34/TIF35) bind to the extreme C-terminal domain (CTD) of PRT1 and to one another, and the largest subunit (TIF32) binds to the N-terminal domain (NTD) in PRT1. The remaining subunit NIP1 binds to TIF32 but not to PRT1. Both eIF5 and eIF1 interact with the NIP1 NTD (Fig. 23) (Valasek *et al.*, 2001; Asano *et al.*, 2001).

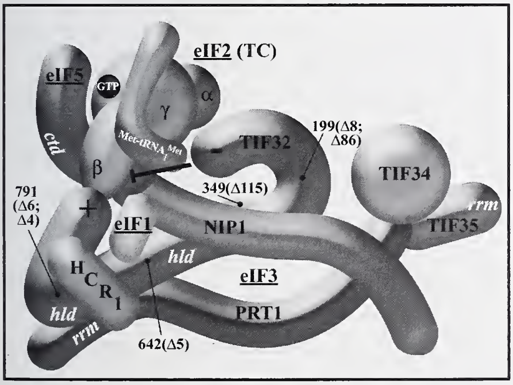

**FIGURE 23**

Schematic representation of the Multifactor Complex comprised of eukaryotic initiation factors, 1, 3, 5, and the eIF2/GTP/Met-tRNAiMet ternary complex.

We demonstrated that the eIF5 CTD contains a conserved motif that can bind simultaneously to the N-terminus of the β-subunit of eIF2 and the NIP1-NTD. These interactions enable eIF5 to bridge an interaction between eIF2 and eIF3 *in vivo*, and a multifactor complex (MFC) containing eIFs 1, 2, 3, and 5 and Met-tRNA$_i^{\text{Met}}$ was purified from cell extracts (Fig. 23). The integrity of the MFC depends on the bridging function of eIF5, which is disrupted by a mutation in the eIF5 CTD (*tif5-7A*). As *tif5-7A* also impairs translation initiation *in vivo* and cell growth, we proposed that the MFC is an important intermediate in translation initiation and that eIF5 plays an important role in initiation complex assembly beyond its conventional function as a GAP (Asano *et al.*, 2001).

To obtain *in vivo* support for our model of the MFC, we incorporated an affinity tag into TIF32, PRT1, and NIP1, the three largest eIF3 subunits, and deleted predicted binding domains in each tagged protein. By purifying and characterizing the mutant subcomplexes, we confirmed all binding interactions predicted by our model and uncovered a new interaction between NIP1 and PRT1. In addition to the confirmed contact between eIF2 and the NIP1 NTD bridged by eIF5, we found that the TIF32 CTD binds to eIF2 directly (Fig. 23) and is required for eIF2-eIF3 association *in vivo*. Overexpressing a form of TIF32 lacking CTD exacerbated the translation initiation defect of the *tif5-7A* mutation, weakening the NIP1/eIF5/eIF2 connection. Thus, the two independent eIF2–eIF3 contacts identified here have additive effects on the efficiency of translation *in vivo*.

Overexpressing the NIP1-NTD sequestered eIF1/eIF5/eIF2 in a defective subcomplex that derepressed *GCN4* mRNA translation (Gcd$^-$ phenotype). *GCN4* translation is normally repressed by its four upstream open reading frames (uORFs). Ribosomes that translate uORF1 and resume scanning reinitiate at uORF4 and fail to reach *GCN4* unless the concentration of TC is reduced by eIF2α phosphorylation or the ability of TC to rebind to ribosomes scanning between uORFs 1 and 4 is impaired by mutation. Under these circumstances, the ribosomes bypass uORF4 and reinitiate at *GCN4* instead. The Gcd$^-$ phenotype of overexpressing the NIP-NTD was enhanced by overexpressing the TIF32-CTD, which also sequesters eIF2 in a distinct subcomplex, and was suppressed by overproducing the constituents of the TC. These results provide the first evidence that physical association with eIF3 promotes binding of TC to 40S ribosomes *in vivo* (Valasek *et al.*, 2002).

### Domains in eIF1A That Interact with eIF2, eIF3, and eIF5B and Promote Ribosome Binding of the Ternary Complex

*Olsen, Savner, Mathew, Zhang, Hinnebusch*

Because of its homology to bacterial translation initiation factor IF1, the eukaryotic factor eIF1A is predicted to bind in the decoding (A) site of the 40S ribosome, and mammalian eIF1A has been implicated in recruitment of TC and ribosomal scanning. We showed previously that yeast eIF1A interacts *in vivo* and *in vitro* with eIF5B, the ortholog of bacterial IF2 that promotes 40S–60S subunit joining *in vitro*. The fact that overexpression of eIF1A exacerbated the growth defect of a strain lacking eIF5B led us to propose that eIF1A's interaction with eIF5B is instrumental in release of eIF1A from the A-site following subunit joining. Consistent with this hypothesis, we have mapped the binding domain for eIF5B to the last 24 residues of the unstructured C-terminus of eIF1A and shown that removal of these residues diminishes translation initiation *in vivo*. A larger C-terminal truncation that removes a predicted $3_{10}$ helix in eIF1A derepresses *GCN4* translation ($Gcd^-$ phenotype), a derepression that can be suppressed by overexpressing the TC. These latter results provide the first evidence that eIF1A promotes TC binding to 40S ribosomes *in vivo*. The unstructured N-terminus of eIF1A interacts directly with eIF2 and eIF3 and is required for optimum translation at a step following TC recruitment, especially at low growth temperatures. We propose a modular organization for eIF1A in which a core ribosome-binding domain is flanked by flexible segments that mediate its interactions with other factors involved in TC recruitment and release of eIF1A at subunit joining.

### Activation of the Intrinsically Defective Kinase Domain in GCN2 by Mutations That Bypass tRNA Binding

*Qiu, Hu, Dong, Hinnebusch*

GCN2 down-regulates general translation and specifically induces GCN4 translation by phosphorylating eIF2α and thereby impeding TC formation. GCN2 contains regulatory domains located both N-terminal and C-terminal to the protein kinase (PK) domain, including a region related to histidyl-tRNA synthetase (HisRS), a C-terminal ribosome-binding and dimerization domain (Cterm), and an N-terminal domain that mediates binding of the positive effectors GCN1 and GCN20. The HisRS-related domain can bind to tRNA *in vitro* and mediates the activation of GCN2 by uncharged tRNA in amino acid–starved cells. The mechanism of kinase activation is not well understood but appears to depend on physical contact between the PK domain and the N-terminal portion of the HisRS region (HisRS-N) bound to uncharged tRNA. In addition, interactions between the PK and Cterm play a role in preventing tRNA binding and kinase activation at low, basal concentrations of uncharged tRNA present in nonstarved cells (Qiu *et al.*, 2001).

To elucidate the mechanism of kinase activation, we isolated two point mutations in the protein kinase (PK) domain, R794G and F842L, that permit strong kinase activity in the absence of tRNA binding. These mutations bypass the requirement for ribosome binding, dimerization, and association with the GCN1/GCN20 regulatory complex, suggesting that all these functions facilitate tRNA binding to wild-type GCN2. While the isolated wild-type PK domain was completely inert, the mutant PK was highly active *in vivo* and *in vitro*. These results identify an inhibitory structure intrinsic to the PK domain that must be overcome on tRNA binding by interactions with a regulatory region, most likely the HisRS-N/tRNA module. As R794 and F842 are predicted to lie close to one another and to the active site, they may participate directly in misaligning active site residues. Consistent with this idea, many wild-type protein kinases contain Gly and Leu residues at the positions corresponding to the *R794G* and *F842L* activating mutations in GCN2. Autophosphorylation of two Thr residues in the activation loop of the kinase domain is required for GCN2 activation. Autophosphorylation of these residues was stimulated by *R794G* and *F842L*, and the autophosphorylation sites remained critical for *GCN2* function in the presence of the activating mutations. Hence, our results imply a two-step mechanism for activation by uncharged tRNA: (1) interaction between the PK domain and HisRS-N/tRNA module elicits a conformational change that corrects the intrinsic defect of the PK domain, leading to (2) autophosphorylation of the activation loop and activation of the eIF2± kinase function of the PK domain (Qiu *et al.*, 2002).

### Multiplicity of Coactivators Required by GCN4p at Individual Promoters *in Vivo*

*Swanson, Qiu, Krueger, Sumibcay, Kim, Zhang, Hinnebusch*

Eukaryotic transcriptional regulation requires sequence-specific DNA binding proteins to activate transcription from target genes that are organized in repressive chromatin. Activators recruit multisubunit coactivators harboring activities that alter chromatin structure and recruit the general transcription factors and RNA polymerase to the promoter. Yeast cells respond to amino acid starvation by inducing the activator of amino acid biosynthetic genes, GCN4. Our previous studies showed that the SAGA, SWI/SNF, and SRB/MED mediator complexes can interact with GCN4 *in vitro* and that mutations in subunits of these complexes decrease activation by GCN4 *in vivo* (Gcn$^-$ phenotype). SAGA contains the histone acetyltransferase (HAT) Gcn5p, which acetylates lysines in the tails of histone H3, plus additional subunits involved in recruiting TATA binding protein (TBP) to the promoter. SWI/SNF is an ATP-dependent chromatin-remodeling complex, and the SRB/MED mediator is associated with RNA polymerase in a holoenzyme complex, transducing the effects of activators and repressors on polymerase function.

We have conducted a comprehensive analysis of coactivator requirements by testing all viable mutants from the *Saccharomyces* Deletion Project for defects in activation by GCN4 *in vivo*. Our data confirm that GCN4 requires SAGA, SWI/SNF, and SRB/MED and identify the key nonessential subunits in these complexes required for activation *in vivo*. Among the numerous histone acetyltransferases examined, GCN5 was uniquely required for activation by GCN4. We also uncovered a strong dependence on the CCR4/NOT complex and significant requirements for RSC and the Paf1 complex, but we could detect no role for ISW1/ISW2 in activation by GCN4. RSC and ISW are ATP-dependent chromatin-remodeling complexes, the Paf1 complex is an alternative form of mediator, and CCR4/NOT has been implicated in controlling TBP binding and holoenzyme function. *In vitro* binding experiments indicate that the GCN4 activation domain interacts specifically with CCR4-NOT and RSC in addition to SAGA, SWI/SNF, and SRB/MED, but not with the Paf1 complex. Chromatin immunoprecipitation experiments show that GCN4 recruits all six of these coactivators to one of its target genes (*ARG1*) in living cells. Deletions of certain SAGA and SRB/MED subunits with strong activation defects *in vivo* disrupt binding of GCN4 to these complexes *in vitro*. In contrast, deletions of other subunits with Gcn$^-$ phenotypes do not reduce coactivator binding to GCN4 and hence may impair a biochemical function of the coactivator at the promoter.

## SELECTED PUBLICATIONS

1. Algire MA, Maag D, Savio P, Acker MG, Tarun SZ Jr, Sachs AB, Asano K, Nielsen KH, Olsen DS, Phan L, Hinnebusch AG, Lorsh JR.. Development and characterization of a reconstituted yeast translation initiation system. RNA. 2002;8:382-397.
2. Asano K, Hinnebusch AG. Protein interactions important in eukaryotic translation initiation. In: McDonald PN, ed. Methods Mol Biol. 2001;177:179-198.
3. Asano K, Phan L, Krishnamoorthy T, Pavitt GD, Gomez E, Hannig EM, Nika J, Donahue TF, Huang HK, Hinnebusch AG. Analysis and reconstitution of translation initiation in vitro. Methods Enzymol. 2002;351:221-247.
4. Asano K, Phan L, Valasek L, Schoenfeld LW, Shalev A, Clayton J, Nielsen K, Donahue TF, Hinnebusch AG. A multifactor complex of eIF1, eIF2, eIF3, eIF5, and $\text{tRNA}_i^{\text{Met}}$ promotes initiation complex assembly and couples GTPase hydrolysis to AUG recognition. Cold Spring Harbor Symp Quant Biol. 2001:66:403-415.

5. Gaba A, Wang Z, Krishnamoorthy T, Hinnebusch AG, Sachs MS. Physical evidence for distinct mechanisms of translational control by upstream open reading frames. EMBO J. 2001;20:6453-6463.
6. Garcia-Barrio M, Dong J, Cherkasova VA, Zhang X, Zhang F, Ufano S, Lai R, Qin J, Hinnebusch AG. Serine 577 is phosphorylated and negatively affects the tRNA binding and eIF2$\alpha$ kinase activities of GCN2. J Biol Chem. 2002;277:30675-30683.
7. Hinnebusch AG. Unleashing yeast genetics on a factor-independent mechanism of internal translation initiation. Proc Natl Acad Sci USA. 2001;98:12866-12868.
8. Hinnebusch AG, Natarajan K. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryotic Cell. 2002;1:22-32.
9. Phan L, Schoenfeld LW, Valasek L, Nielsen KH, Hinnebusch AG. A subcomplex of three eIF3 subunits binds eIF1 and eIF5 and stimulates ribosome binding of mRNA and $tRNA_i^{Met}$. EMBO J. 2001;20:2954-2965.
10. Qiu H, Dong J, Hu C, Francklyn CS, Hinnebusch AG. The tRNA binding moiety in GCN2 contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation. EMBO J. 2001;20:1425-1438.
11. Qiu H, Hu C, Dong J, Hinnebusch AG. Mutations that bypass tRNA binding activate the intrinsically defective kinase domain in GCN2. Genes Dev. 2002;16:1271-1280.
12. Sattlegger E, Hinnebusch AG. Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid starved cells. EMBO J. 2000;19:6622-6633.
13. Valasek L, Hasek J, Nielsen KH, Hinnebusch AG. Dual function of eIF3j/Hcr1p in processing 20 S pre-rRNA and translation initiation. J Biol Chem. 2001;46:43351-43360.
14. Valasek L, Nielsen KH, Hinnebusch AG. Direct eIF2-eIF3 contact in the multifactor complex is important for translation initiation in vivo. EMBO J. 2002;21:5886-5898.
15. Valasek L, Phan L, Schoenfeld LW, Valaskova V, Hinnebusch AG. Related eIF3 subunits TIF32 and HCR1 interact with an RRM in PRT1 required for eIF3 integrity and ribosome binding. EMBO J. 2001;20:891-904.

## EPIGENETIC GENE SILENCING

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Masaya Oki, Ph.D., *Postdoctoral Fellow*
Lourdes Valenzuela, Ph.D., *Postdoctoral Fellow*

Research in this laboratory is devoted to understanding the mechanisms by which entire regions of the genome are rendered inaccessible to transcription and recombination. The laboratory uses genetic analysis, coupled with biochemical fractionation and reconstitution experiments, to explore the issues of genome accessibility. Silencing of genomic domains requires a complex series of interactions between inactivation centers called silencers and numerous repressor proteins. The silencers recruit repressor protein complexes composed of the Sir proteins, which then interact with histones in nucleosomes to form a chromatin domain, a domain that is both inaccessible and inert to various cellular processes. We are currently focusing on these proteins and their interactions with the histones to understand, in molecular detail, the mechanism by which silencing is effected.

### Histone Variants and Cell Cycle Progression

*Dhillon*

It has been previously demonstrated that *RAP1*, *ORC*, and *SIR1* are necessary to establish the silent state. Sir1p acts as an intermediary molecule between the proteins bound to the silencer element (Rap1p and Orc) and the proteins involved in maintaining the repressed state: Sir2, Sir3, Sir4, and the histones.

The role of histone variants in gene expression is poorly understood. To understand the function of Sir1 in silencing in *S. cerevisiae*, we generated yeast cells with mutations in this protein that were unable to silence even after Sir1 was recruited to the silencer, presumably owing to a weakened interaction between Sir1p and the other proteins involved in silencing. Overproduction of the histone variant Htz1p in the *sir1* mutant could compensate for its weakened interaction (Dhillon and Kamakaka, 2000). We are currently interested in understanding the many roles of this protein in the cell. Classical molecular genetic and biochemical experiments are under way to identify the domains of Htz1 involved in cell cycle progression, determine the response to drugs such as hydroxyurea and benomyl, and identify the proteins that interact with Htz1 to function in the cell.

### Identification and Characterization of Sir Protein Complexes Involved in Silencing

*Gangadharan*

Our main goal is to determine how Sir proteins function to form silenced domains. Genetic studies have revealed that distinct combinations of the Sir protein complexes repress multiple loci. Silencing at these loci requires Sir2p, which possesses histone deacetylase activity. In addition, Sir2p is the only Sir protein to have additional homologs in yeast (Hst1-4p) and the only Sir protein conserved throughout evolution. We are purifying Sir2p-containing protein complexes from yeast cells to identify and characterize individual components within these complexes and their associated enzymatic activities. We have also initiated studies on the reconstitution of silenced chromatin by using these purified complexes and histones in nucleosomes (Ghidelli *et al.*, 2001). Our ability to mimic the silenced state *in vitro* will be an important gauge of our current understanding of transcriptional silencing.

We will employ reverse genetics to determine the function of any novel polypeptides associated with these complexes. We will also perform experiments to analyze the nature of the interactions between these Sir protein complexes and histones in nucleosomes. This analysis will involve binding studies with purified Sir complexes and positioned nucleosomes in arrays, followed by DNaseI foot-printing and protein-protein cross-linking and sedimentation analyses. Long-term goals call for studies on the regulation of these enzymes within the cell and *in vitro* studies aimed toward the eventual development of specific inhibitors for these enzymes that may have therapeutic value.

### Role of Nuclear Architecture in Silencing

*Oki, Valenzuela; in collaboration with Ito*

We are also interested in understanding the mechanism by which silenced chromatin domains are restricted to specific regions along the DNA fiber. Eukaryotic chromosomes are organized into discrete domains that are delimited by domain boundaries. We have demonstrated that a specific tRNA gene mediates barrier functions at the *HMR* locus. The proteins that are required to prevent the spread of heterochromatin into neighboring euchromatin have also been identified (Donze and Kamakaka, 2000). Our results suggest that barrier activity may arise from an underlying competition between chromatin remodeling and silencing at the interface of euchromatin and heterochromatin. In our ongoing studies on yeast barriers, we have used genetic screens both to isolate other DNA elements from yeast and other eukaryotes that act as barriers and to identify additional proteins from yeast and other eukaryotes that possess barrier activity. These studies will help delineate general principles of barrier activity in all eukaryotes. In addition, given that other transcriptionally inactive genes such as the *SMK1* loci in *S. cerevisiae* are also packaged into repressive chromatin domains, we can address whether the repressors that function at this locus are restricted by the presence of specific barriers and whether these elements function by similar mechanisms.

### Analysis of Sir2p in Other Eukaryotes

*Haldar*

A complete understanding of transcriptional repression requires the analysis of several unrelated loci in different and distinct systems so that salient principles of repression can be distinguished from organism- and locus-specific variation. Silencing of chromatin domains in *S. pombe* shows many similarities to heterochromatin formation and position effect variegation in other eukaryotes such as *S. cerevisiae* and *Drosophila*. Functional homologs of the various *S. cerevisiae* genes that affect repression are being identified in *S. pombe* to allow the purification and characterization of protein complexes containing such proteins. This work will be coupled with mechanistic studies on nucleosomal binding in this organism.

## SELECTED PUBLICATIONS

1. Dhillon N, Kamakaka RT. A histone variant Htz1p and a Sir1-like protein Esc2p mediate silencing at *HMR*.. Mol Cell. 2000;6:769-780.
2. Donze D, Kamakaka RT. RNA Polymerase III and RNA Polymerase II promoter complexes are heterochromatin barriers in *Saccharomyces cerevisiae*. EMBO J. 2001;20:520-531.
3. Ghidelli S, Donze D, Dhillon N, Kamakaka RT. Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities. EMBO J. 2001;20:4522-4535.

## COLLABORATOR

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# MOLECULAR MECHANISM OF NEURONAL CONNECTIVITY

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Using the *Drosophila* visual system as a model, our laboratory is studying the molecular mechanisms by which neurons established specific connections during development. The fly retina contains three classes of photoreceptor neurons (R1-6, R7, and R8), each responding to a specific spectrum of light, and connecting to a specific layer in the brain (Fig. 24). We focus on the layer-specific targeting of R7 neurons, which are essential for visual function and, moreover, amenable to genetic manipulation. By using visual-driven behavior assays, we previously identified N-cadherin (Ncad) and the receptor tyrosine phosphatase LAR because of their requirement for R7 target selection (Lee *et al.*, 2001). Mosaic analysis showed that Ncad and LAR are required "cell-autonomously" in R7 neurons for selecting the proper target layer. To understand how Ncad regulates R7 target selection, we developed a genetic method to analyze single Ncad mutant R7 axons during development. Our data suggest that Ncad provides adhesive interactions between R7 growth cones and their synaptic partners. Furthermore, we have uncovered the striking molecular diversity of Ncad, which is generated by alternative splicing. We are testing the hypothesis that combinatory use of Ncad isoforms forms a synaptic code that matches pre- with post-synaptic partners.

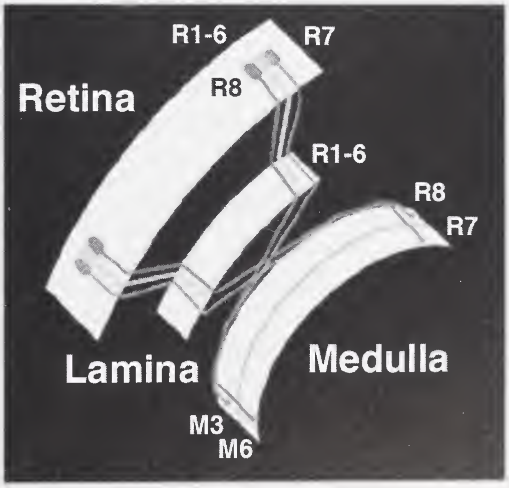

**FIGURE 24**
Connectivity of *Drosophila* retina.

### Molecular Diversity of N-Cadherin

*Lee, Chiba, Hsu*

Known as the chemoaffinity theory as proposed by Roger Sperry 40 years ago, the possibility that surface receptors exist to serve as molecular tags directing neuronal connectivity has remained a matter of great interest to researchers. Given that the number of synaptic connections far exceeds the predicted number of surface receptors, it is unlikely that the correspondence between pre- and post-synaptic partners mediated by such surface receptors represents a simple one-to-one relationship. It has been proposed that the combinatorial use of surface receptors and the differential regulation of receptor function operate as additional mechanisms for directing specific connections. Furthermore, the receptor repertoire can be expanded by alternative splicing, as observed in several receptors such as vertebrate protocadherin CNR (cadherin-related neuronal receptor) and insect Dscam (Down syndrome cell adhesion molecule). The identification of Ncad as the key regulator of R7 targeting and the discovery of multiple Ncad isoforms generated by alternative splicing allow us to examine directly how these mechanisms contribute to connection specificity.

As removing Ncad results in only about 75 percent R7 axon mistargeting (Lee *et al.*, 2001), we suspected the existence of an additional molecule(s) that is functionally redundant with Ncad. Genomic sequence analysis revealed a cadherin gene (designated *Ncad2*) located next to the *Ncad* gene (Fig. 25). The *Ncad2* gene is smaller than the *Ncad* gene and appears to result from partial duplication of the *Ncad* gene. RNA transcript analysis indicated that *Ncad2* is expressed in the developing eye disk. In addition, mature *Ncad2* transcripts consist of two alternative spliced forms, one of which contains exon 9 and encodes a type I receptor that is similar to *Ncad* while the other form does not contain exon 9 and encodes a secreted molecule. The soluble form of cadherins has been shown to disrupt cell-adhesion. These two *Ncad2* forms are present in about equal proportion in the eye disk mRNA pool; in the mRNA of the whole animal, the receptor type is predominant. Thus, this alternative splicing appears to be regulated in a tissue-specific fashion. We are currently generating *Ncad2* mutants for analyzing *Ncad2* function *in vivo.*

We detected the striking molecular diversity of Ncad generated by alternative splicing. The genomic sequence analysis revealed that the *Ncad* locus contains three pairs of exons (exon7–exon7', exon13–exon13', and exon18–exon18') in modular arrangement (Fig. 25). RNA transcript analysis indicated that these exon pairs are used in a mutually exclusive manner, i.e., each mature transcript contains only one of the two alternative exons. All six exons are used in developing eye tissue, although exons 7, 13, and 18' are used predominantly in the eye disk. Using these three "exon modules" in a combinatorial fashion, the *Ncad* locus is capable of encoding eight isoforms, which each share the same molecular architecture but has a unique amino acid sequence. Similar molecular diversity generated by modular arrangement of alternative exons has been observed in other receptors and might be a general mechanism for neuronal diversity and connection specificity.

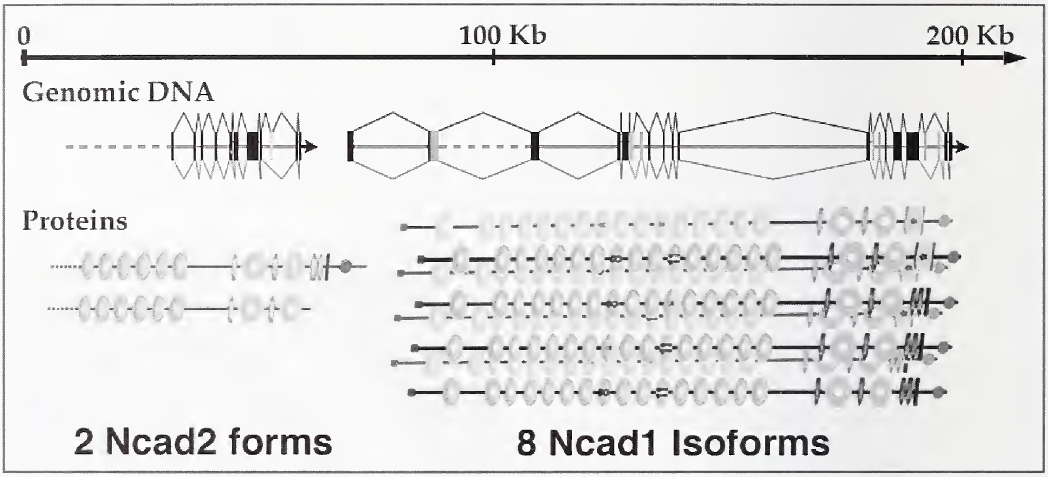

**FIGURE 25**

N-cadherin loci contain constant regions and variable regions.

The notion that the different Ncad isoforms might have distinct functions was first suggested by protein sequence analysis and later verified by cell aggregation assay. Exons 7 and 7' encode the C-terminal half of the eighth cadherin repeat and N-terminal half of the ninth cadherin repeat, and exons 13 and 13' encode the eleventh and twelfth cadherin repeats in a similar manner. Exons 18 and 18' encode one and one-half of the EGF repeat and one-half of the transmembrane region. Interestingly, we found nonconservative amino acid changes in two regions that potentially mediate calcium binding and homophilic interactions, suggesting that these isoforms might have different adhesive activity. To test such a possibility, we expressed individual isoforms in S2 cells and assayed their ability to induce cell aggregation. Our preliminary data showed that the isoforms encoded by exon 7 but not exon 7' are capable of mediating homophilic interaction. In contrast, the region of Ncad encoded by exon13 and exon13' does not significantly affect Ncad's ability to induce cell aggregation. We are currently testing whether these isoforms can mediate heterophilic, or graded, interaction.

## Cellular and Molecular Mechanism of R7 Target Selection

*Lee, Khan*

Like the vertebrate cortex, the medullar neuropil of *Drosophila* brains is laminated, with each layer (or lamina) receiving input from different afferents. R7 neurons connect to the deepest layer of the external medulla neuropil (designated M6) while the lamina interneurons and R8 connect to the more superficial layers, M1-5 and M3, respectively. To elucidate the mechanism by which R7 growth cones choose the appropriate target layer, we first characterized the cellular events occurring during the development of R7 target connections. To resolve the complex cellular interactions involved, we genetically ablated the cells of interest and examined the effects on R7 target selection. Finally, we performed developmental mosaic analyses on mutants with R7 targeting defects, allowing us to assign molecules to specific types of cells and cellular recognition events in which these molecules function. The analyses revealed that R7 target selection proceeds in three consecutive stages, each of which involves different cellular and molecular interactions.

During development, R8, R7, and laminal interneurons (LNs) sequentially innervate the medullar neuropil. R8s differentiate first and extend their axons into the medulla. Approximately 12 hours later, R7 axons project, along the R8 axon shafts, into the medulla, followed shortly thereafter by the LNs. The first stage of R7 target selection involves R7 growth cones defasciculating from R8 axons, thereby allowing R7 growth cones to advance even deeper into the medulla. The separation of R7 and R8 growth cones requires LNs whose axons follow R7 and project into the region between the R8 and R7 growth cones. Genetic ablation of LNs, using an eye-specific allele of hedgehog mutants (*hh1*), blocks the separation of R7 and R8 growth cones. Although the interaction between LNs and R7 (or R8) appears to be critical, the molecular nature of these afferent-afferent interactions is not currently understood.

In the second stage, it is likely that the R7 growth cones enter the target layer by recognizing environmental cues provided by their target neurons. This process requires Ncad function. Removing Ncad in single R7 neurons causes premature arrest of R7 growth cones at the intermediate layer (M4-5) instead of their entering the presumptive R7 recipient layer (M6). R7 target neurons sprout dendritic processes in this layer and express Ncad. *In vitro*, Ncad is capable of mediating homophilic interaction. We favor the view that Ncad provides adhesive interaction between R7 growth cones and their targets.

Once R7 growth cones reach the presumptive R7 recipient layer, they stop and undergo a conformational change from a spear-like structure into an expanded conformation. This morphological change signifies the beginning of the third stage, the stabilization phase. In *LAR* mutants, R7 axons project into the correct layer at earlier stages but later retract to the R8-recipient layer, suggesting that LAR is required for stabilizing the interactions between R7 and their targets (Clandinin *et al.*, 2001). In vertebrates, LAR has been suggested to up-regulate N-cadherin–mediated homophilic adhesion by dephosphorylating β-catenin. We envision that LAR stabilizes R7 target interaction by up-regulating Ncad activity in R7 growth cones.

Our analyses have dissected R7 target selection into a series of complex cellular and molecular interactions that involve afferent-afferent interactions as well as afferent-target interactions executed in a temporally and spatially controlled fashion. At the molecular level, the Ncad-based adhesion system appears to play a key role in R7 target selection. The targeting specificity, we believe, is achieved by both differential modulation of Ncad adhesive activity in different growth cones and combinatorial use of Ncad and Ncad2 isoforms as well as by other surface receptors. It is likely that other determinants of connection specificity remain to be discovered. The developmental analysis presented here provides a conceptual framework upon which the molecular details can be built.

## SELECTED PUBLICATIONS

1. Clandinin TR, Lee CH., Herman T, Lee RC, Yang AY, Ovasapyan S, Zipursky SL. *Drosophila* LAR regulates R1-R6 and R7 target specificity in the visual system. Neuron. 2001;32:237-248.
2. Lee CH, Herman T, Clandinin TR, Lee R, Zipursky SL. N-cadherin regulates target specificity in the *Drosophila* visual system. Neuron. 2001;30:437-450

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# MITOTIC REGULATION IN HIGHER EUKARYOTES BY RaN AND SUMO-1

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Our goal is to understand how cells send and interpret signals that coordinate events during the cell cycle. Our studies concentrate on two closely linked biochemical pathways, both of which have been genetically implicated in the regulation of mitosis: the Ran GTPase pathway and the SUMO-1 conjugation pathway. Ran-GTP acts as an important indicator for the spatial organization of cells during interphase and mitosis while SUMO-1 regulates Ran through the modification of its GTPase-activating protein, RanGAP1. We are currently examining how these pathways are regulated and how they in turn contribute to the control of mitotic progression.

## The Small Ubiquitin-Like Modifier SUMO-1 in Higher Eukaryotes

*Anan, Ayaydin, Azuma, Breaux, Cavenagh, Hang, Joseph, Quimby, Tan, Dasso*

SUMO-1 is a ubiquitin-like protein that can be conjugated to other proteins in a manner similar to ubiquitin conjugation. Our goal is to understand the function and regulation of SUMO-1 at a molecular level, with a particular focus on the mitotic roles of SUMO-1 and how SUMO-1 controls the Ran GTPase–activating protein, RanGAP1.

SUMO proteases are required for the processing of SUMO-1 before conjugation and for deconjugation of SUMO-1 from other proteins. Two SUMO proteases have been described in budding yeast, Ulp1p and Ulp2p/Smt4p. Ulp1p is concentrated near the nuclear periphery and interacts with nuclear pore components, as demonstrated in two-hybrid assays. ULP1 is an essential gene, and temperature-sensitive Ulp1p mutants arrest at the G2/M transition of the cell cycle. In mammals, there are at least seven SUMO protease family members. We have been studying one member of this family in particular: SENP2. We found that full-length human SENP2 associates with nuclear pores in a manner similar to Ulp1 in yeast. The association occurs exclusively with the nuclear face of the pore and requires sequences near the N-terminus of SENP2. We also found that SENP2 binds specifically to Nup153, a nucleoporin localized to the nucleoplasmic face of the nuclear pore, and that the association requires the same domain of SENP2 that mediates its targeting *in vivo*. Remarkably, a mutant SENP2 protein that is unable to bind to Nup153 is significantly more effective in promoting deconjugation of SUMO-1–conjugated species, indicating that localization of SENP2 to the nuclear pore plays an important role in spatially restricting the activity of this enzyme.

Ran-GTP has an important role in regulating the organization of the cell during both interphase and mitosis (see below). Given this role, knowledge of the distribution of Ran regulators will be essential for understanding the control and function of this pathway. In metazoans, RanGAP1 is conjugated with SUMO-1. SUMO-1 modification causes RanGAP1 to associate with RanBP2, a large nuclear pore protein, and with Ubc9, the E2 enzyme for SUMO-1 conjugation. We have examined the behavior of RanGAP1 during mitosis and found that RanGAP1 associates with mitotic spindles and is particularly concentrated at foci near kinetochores. Association with kinetochores appears soon after nuclear envelope breakdown and persists until late anaphase but is lost coincident with nuclear envelope assembly in telophase. A mutant RanGAP1 protein lacking the capacity to be conjugated to SUMO-1 no longer associated with spindles, indicating that conjugation is essential for mitotic localization of RanGAP1. RanBP2 co-localizes with RanGAP1 on spindles, suggesting that a complex between these two proteins may be involved in mitotic targeting of RanGAP1. Our findings have shown for the first time that SUMO-1 conjugation is required for mitotic localization of RanGAP1 and indicate that a major role of SUMO-1 conjugation to RanGAP1 may be the spatial regulation of the Ran pathway during mitosis.

### Mitotic Roles of Ran GTPase

*Arnaoutouv, Dasso*

We are interested in the systems required for maintenance of mitotic arrest. To complete division and exit mitosis, cells must degrade the cyclin B protein and thereby inactivate the cdc2 kinase. Cyclin B is targeted for degradation by the anaphase-promoting complex (APC/C) with the help of FZY, an adapter protein whose binding to APC/C is necessary for cyclin ubiquitination. After ubiquitination, cyclin B is rapidly degraded by proteasomes. Cells can be arrested in M phase in response to developmental cues (i.e., *Xenopus laevis* frog eggs remain arrested in second meiotic metaphase until fertilization; called CSF arrest) or in response to checkpoint signals (i.e., most vertebrate cells arrest in M phase if one or more unattached kinetochores are present; called checkpoint arrest). Both types of arrest can be mimicked in extracts prepared from *Xenopus* eggs. Addition of calcium eliminates CSF arrest in egg extracts. The mechanism of this release is very similar to the calcium wave triggered by fertilization that promotes destruction of cyclin B and CSF activities and releases the eggs into first interphase. A microtubule-dependent checkpoint can be generated in egg extracts by adding the microtubule destabilizing agent nocodazole and chromosomes. This microtubule-dependent checkpoint is not sensitive to calcium.

To understand the molecular mechanisms that are responsible for generating the two types of arrest, we followed the fate of an I-125–labeled fragment of cyclin B1 in *Xenopus* egg extracts and observed *in vitro* reactions with purified components reconstructing ubiquitination by APC/C and FZY, deubiquitination by isopeptidases, and degradation by proteasomes. We found that exit from mitosis, and therefore degradation of cyclin B, is always accompanied by activation of ubiquitination through APC/C while isopeptidase and proteasomal activities are not changed. After checkpoint activation, APC/C activity was blocked by sequestration of FZY into an inactive complex with checkpoint proteins, thus preventing association of FZY with APC/C. Cyclin B degradation could be restored in checkpoint-arrested extracts with physiological levels of baculovirus-produced FZY. While CSF arrest is also mediated by inhibition of APC/C, we found that it cannot be restored by the addition of FZY. To the contrary, our data currently suggest that CSF arrest results from direct inhibition of APC/C by phosphorylation. Taken together, our results indicate that different types of mitotic arrest use different approaches to stabilize Cyclin B.

We are also interested in examining how Ran interacts with mitotic regulatory pathways. Ran is a GTPase that is required for nuclear transport, cell cycle control, mitotic spindle formation, and post-mitotic nuclear assembly. The cytosolic GTPase-activating protein RanGAP1 and the chromatin-bound nucleotide exchange factor RCC1 regulate Ran. The distribution of Ran-GTP provides important spatial information that directs cellular activities throughout the cell cycle. During interphase, the localization of

RCC1 and RanGAP1 predicts that nuclear Ran is bound to GTP and cytosolic Ran is bound to GDP. This compartmentalization determines the direction of nuclear transport by promoting the loading and unloading of transport receptors in a manner that is appropriate to the nucleus or cytosol. In mitosis, chromatin-bound RCC1 protein generates a high concentration of Ran-GTP in the vicinity of the chromosomes. In a manner that is essential for the formation of a correct bipolar spindle, Ran-GTP acts to stabilize microtubules (MTs) near the chromosomes. We have observed that the addition of bacterially expressed RCC1, but not an inactive RCC1 mutant, overrides the spindle-assembly checkpoint in a dose-dependent manner. We have further documented that exogenous RCC1 changes the behavior of checkpoint pathway components. Notably, increased concentrations of RCC1 do not release extracts from CSF-mediated arrest. Our results suggest that the mitotic function of Ran GTPase lies not only in formation of the microtubule spindle but also in regulating the checkpoint pathway.

## SELECTED PUBLICATIONS

1. Azuma Y, Dasso M. A new clue at the nuclear pore RanBP2 is an E3 enzyme for SUMO1. Dev Cell. 2002;2:130-131.
2. Dasso M. The Ran GTPase theme and variations. Curr Biol. 2002;12:R502.
3. Hang J, Dasso M. Association of the human SUMO-1 protease SENP2 with the nuclear pore. J Biol Chem. 2002;277:19961-19966.
4. Joseph J, Dasso M. Cellular roles of the Ran GTPase. In: Hamm H, ed. Handbook of cellular signaling. San Diego: Academic Press, 2002; in press.
5. Joseph J, Tan SH, Karpova TS, McNally JG, Dasso M. SUMO-1 targets RanGAP1 to kinetochores and mitotic spindles. J Cell Biol. 2002;156:595-602.

# MOLECULAR GENETICS OF RETROTRANSPOSONS

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Retroelements are a large class of genetic elements that multiply by the reverse transcription of an RNA intermediate. The resulting cDNA is incorporated into the genome of host cells. In eukaryotes, the success of long terminal repeat (LTR)–containing retroelements has led to replication mechanisms that are conserved among diverse families of retrotransposons and retroviruses. Given that LTR-retrotransposons exist in yeast, we have applied powerful techniques of yeast genetics to basic questions about the function of LTR-retroelements, specifically the molecular mechanisms of reverse transcription, transport of the retrotransposon Tf1 into the nucleus, integration of Tf1 cDNA into the host genome, and the residues that contribute to the specialized steps of reverse transcription. Our study of nuclear import is motivated by the finding that mutations in a specific factor of the nuclear pore blocks import of Tf1 virus-like particles without altering the growth of the cells. Thus, it may be possible to inhibit the infection of specific retroviruses by blocking their import into the nucleus. The process of integration is significant because of its impact on the host genome. Tf1 integration serves as a model for retroviruses and retrotransposons of humans and how they choose which host sequences to disrupt.

## Specific Contribution of the Primer Grip in the RNase H of Reverse Transcriptase to Removal of the Plus Strand Primer from the 5' End of the cDNA

*Atwood-Moore, Khoo, Levin*

The complete process of reverse transcription requires the recognition and removal of specialized primers for both the plus and minus strands. Little is known about which domains of reverse transcriptase (RT) recognize and remove the primers from the ends of the cDNA. To uncover the amino acids that mediate the individual events or reverse transcription, we mutagenized the complete sequence of Tf1 RT and inserted it into a plasmid form of Tf1 that is used to measure transposition activity in *S. pombe*. We isolated 3,000 strains with mutations in RT that inhibited transposition. Thirty four versions of Tf1 with single amino acid substitutions generated high levels of cDNA products. Surprisingly, one-third of the mutants produced normal levels of full-length, double-stranded cDNA, many of which clustered within a segment of the RNase H domain of RT. A recent demonstration of the importance of this region of RNase H involved a crystallographic study of HIV RT that found that the domain of interest in the RNase H, dubbed the primer grip, interacts directly with the nucleotides of the polypurine tracts (PPT). Both the position and sequence of the primer grip residues in HIV correspond well with the domain we identified in the RNase H of Tf1. We tested the possibility that the mutations resulted in defective transposition as a consequence of altered PPT processing. The change in cDNA sequences at their termini by as little as one or two nucleotides would have a drastic impact on integration catalyzed by integrase (IN). The mutations produced five-fold increases in the levels of cDNA that could not be integrated because the PPT was not removed. Thus, our screen for mutations in RT that make full-length cDNA identified the primer grip of RNase H as contributing to the removal of the PPT RNA from the 5' end of the plus strand LTR. Surprisingly, the ability to process the PPT is specific and is not required for the other steps in the pathway of reverse transcription.

### Mediation of Particle Formation and Reverse Transcription in *S. pombe* by the Gag Protein of Tf1

*Teysset, Kim, Levin; in collaboration with Dang*

The Gag proteins of LTR-retrotransposons and retroviruses assemble into the shell of virus and virus-like particles, with the formation of such particles required for reverse transcription. As a result of its role in particle formation, Gag is responsible for the size of virions and the packaging of the other components of the particle such as the mRNA, RT, and IN. Although Tf1 encodes functional protease, RT, and IN (all proteins known to be necessary, respectively, for protein processing reverse transcription, and integration), the function of the Tf1 Gag protein has yet to be determined. Unraveling that function is significant because the sequence of Tf1 Gag lacks any of the conserved motifs associated with other Gag proteins.

To test whether the Gag of Tf1 was required for particle formation, packaging of mRNA, and reverse transcription, we generated four versions of Tf1 with deletions of eight amino acids in the hydrophilic domains of the Gag. Each mutation significantly reduced transposition. While most N-terminal and C-terminal deletions did not reduce levels of Gag, RT, or IN, the two central deletions did cause severe reductions in Gag. The analysis of cDNA produced by the mutant elements demonstrated that mutants with low levels of Gag were unable to accumulate cDNA. While both the N- and C-terminal mutations generated normal levels of Gag, the C-terminal mutation significantly reduced levels of cDNA. We investigated the role of Gag in packaging Tf1 mRNA by subjecting the virus-like particles to high concentrations of nuclease. Particles produced by wild type and the element with the N-terminal deletion in Gag protected the Tf1 mRNA from degradation while the other three mutations protected the Tf1 mRNA much less, which is consistent with a role of Gag in forming a protective particle. In the case of the C-terminal mutation, the lack of RNA protection by a deletion that expresses normal levels of Gag identified residues necessary for mRNA packaging.

We tested directly the role of Gag in particle formation by visualizing with electron microscopy sections of cells expressing Tf1. In cells expressing wild-type Tf1, we could occasionally detect large arrays of 30 nanometer particles, but never in cells that lacked Tf1 expression. In addition, we never observed particles in the cells with the mutations that inhibited Gag expression. The results indicate that Gag is required for particle formation. More important, Tf1 with a frameshift expressing only Gag was able to produce particles. Thus, as is the case for retroviruses, Gag of Tf1 is sufficient for the formation of particles. We conclude that the Gag of Tf1 is required for transposition and functions as the Gag of retroviruses. The central deletions showed that particles cannot form without Gag and the Tf1 mRNA cannot be packaged. The deletion near the C-terminus of Gag allowed particles to form by identified residues required for mRNA packaging. Finally, the deletion at the N-terminus of Gag did not alter protein levels or reverse transcription. Instead, the mutation likely reduced transposition because it removed the nuclear localizing signal (NLS) that is required for import of Tf1 protein and cDNA into the nucleus.

### Sequences of the *S. pombe* Genome Selected for Integration of Tf1

*Bowen, Levin; in collaboration with Singleton, Wood*

Recognizing that the process of integration has the potential to disrupt genes of the host, we sought to understand the balance between the ability of the transposon to insert into the host genome versus the efforts of the host to maintain its viability. Much of what is known about the insertion sites of LTR-retrotransposons indicates that the process of integration is specifically controlled to avoid the disruption of host genes. In fact, all five transposons of *Saccharomyces cerevisiae* select sites for integration that lack coding sequences. Much less is known about the interactions between the genome of *S. pombe* and its transposons. The recent completion of the genome sequence of *S. pombe* allowed us to study the full set of transposon sequences and their relationship to the host genes.

We conducted a comprehensive search of the genome sequence of *S. pombe* for transposon-related sequences. Surprisingly, the only transposons revealed by the search were related to the Tf1/Tf2 family of LTR retrotransposons. We identified no complete copies of Tf1 and only 13 full-length copies of Tf2. Single LTRs result from the removal of full-length elements by homologous recombination. There were 182 single LTRs in the genome and those with the full-length elements constitute 0.8 percent of the *S. pombe* genome. The single LTRs provide important information about the transposon history of the host genome. Examination of all the LTRs revealed that each element was located within intergenic regions of sequence. Surprisingly, 96 percent of the insertions were in intergenic regions that included pol II promoters, thereby accounting for a significant bias in that pol II promoters are found in only 53 percent of the intergenics. We confirmed the association between pol II promoters and insertion sites by compiling distances between LTR insertions and the nearest ORF. Eighty-four percent of the insertions were closer to a 5' end of an ORF than a 3' end. Moreover, most of the insertions clustered within 200 nucleotides of the start codon.

### Role of Interaction between the Chromodomain of Tf1 IN and Histones with a Modification in Insertion of Tf1 cDNA Adjacent to pol II Promoters

*Nam, Levin; in collaboration with Grewal*

Proteins with conserved motifs dubbed chromodomains are often associated with heterochromatin. Recently, the chromodomain-containing proteins HP1, suVAR(3-9), Clr4p, and Swi6p were shown to generate heterochromatin by binding to specific nucleosomes on DNA. The binding results from an interaction between the chromodomain and histone H3, which is methylated specifically at lysine 9. Interestingly, Malik and Eickbush discovered a sequence at the C-terminus of the Tf1 IN that was similar to a chromodomain. We made mutations in the conserved residues of the putative chromodomain and found that they greatly reduced the transposition activity of Tf1. Experiments conducted by the laboratory of Shiv Grewal demonstrated that, in the *S. pombe* intergenic regions of pol II genes, histone H3 was not methylated at lysine 9 but instead had a high level of methylation at lysine 4, with peaks of lysine 4 methylation near pol II promoters, suggesting that the chromodomain of Tf1 IN may target insertion into intergenic sequences by associating with histone H3 methylated at lysine 4. In collaboration with this laboratory, we tested this model by measuring transposition activity in a strain that was deleted for *set1,* the methylransferase responsible for the methylation of H3 at lysine 4. The deletion rendered methylation of lysine 4 undetectable. More important, the transposition activity of the strain was significantly reduced compared with strains with the wild-type *set1*.

### Tf1 Residues in Gag that Regulate Nuclear Localization

*Kim, Levin*

As in the case for HIV, Tf1 must translocate into the nucleus the proteins and cDNA of its preintegration complex (PIC) before it integrates the cDNA into the host genome. In previous experiments, we identified a component of the nuclear pore that is required for import of Tf1 into the nucleus. The function of the protein Nup124p shows specificity in that its absence disrupts import of Tf1 but does not reduce import of other proteins. Interestingly, the nuclear localization of Gag occurs only when cultures reach stationary phase.

We tested whether residues adjacent to the NLS of Gag regulate the NLS activity. We made five mutant transposons, each with sequential tracts of four amino acids downstream of the NLS replaced with stretches of four alanines. All five versions of Tf1 transposed significantly less than the wild type, but all five mutants made normal amounts of Gag. We showed that two of the mutants (position IV and V) did not complete reverse transcription, indicating that residues in the N-terminus of Gag contributed to reverse transcription. Deletions at positions I, II, and III did not reduce levels of reverse transcription, and the defects in transposition caused by deletions I, II, and III were not caused by changes in protein levels or reduced reverse transcription. Thus, we investigated the possibility that the mutations altered the function of the NLS. In four of the five alanine mutants (mutant positions I through IV), induced for transposition, localization of Gag in the nucleus was significantly reduced, indicating that the residues deleted at positions I, II, and III do contribute to the function of the NLS. Surprisingly, the mutation at position V caused a defect in the regulation of import that allowed Gag to localize in the nucleus even during log phase growth. One explanation is that the mutation V disrupts the Gag-Gag interactions necessary for particle formation, which, in turn, could allow import of a nonparticle form of Gag by a process different from the stationary phase mechanism. Alternatively, deletion V could remove a site of post-translational modification that inhibits import.

### Role of Tf1 t-SNARE Syntaxin in Transport into the Nucleus

*Nam, Peters, Kim, Levin*

The life cycle of Tf1 is very similar to that of retroviruses and has therefore been used as a model system for studying the mechanism of retrovirus propagation. To understand the host functions required for the propagation of LTR elements, we mutagenized a culture of *S. pombe* and screened for mutants that were severely defective for Tf1 transposition. Previous characterization of the strains revealed that mutations in the nuclear pore factor Nup124p and the histone deacetylation factor Sin3p decreased transposition frequencies ten-fold, a reduction that is attributable to defects in the transport of Tf1 into the nucleus. We have now identified a mutation in a newly identified host gene, *psy1*, that reduces Tf1 transposition more than 40-fold. The gene encodes a t-SNARE protein with 65 percent sequence similarity to the homologs of syntaxin in *S. cerevisiae*. Syntaxin proteins reside in membrane structures and promote vesicle trafficking by mediating the fusions of vesicles with target membranes. It is therefore likely that Psy1p mediates some form of vesicle traffic in *S. pombe*. Recent work by Kanamura and Shimoda demonstrated that Psy1p in *S. pombe* is a plasma membrane t-SNARE.

We investigated the role of Psy1p in transposition by testing the effect of the *psy1-1* mutation on the intermediates of Tf1. The mutation did not reduce the levels of production of either Tf1 proteins or cDNA. The data indicate that the defect in transposition occurs after reverse transcription. We tested whether the Tf1 cDNA produced by the mutant strain was transported into the nucleus. The *psy1-1* allele caused a dramatic reduction in the homologous recombination that occurred between Tf1 cDNA and a plasmid copy of Tf1, suggesting that, although cDNA is produced, it is not transported into the nucleus. To test whether the mutation in *psy1-1* altered the import of Tf1 protein, we determined the cellular localization of Gag. While Gag localized in the nucleus of wild-type cells, the *psy1-1* mutation caused Gag to remain in the cytoplasm, indicating that a late step in transposition, potentially transport to the nuclear envelope, requires a specific class of vesicle traffic.

## SELECTED PUBLICATIONS

1. Levin H. Newly identified retrotransposons of the gypsy/Ty3 class in fungi, plants, and vertebrates. In: Craig N, ed. Mobile DNA II. Washington, DC: American Society of Microbiology Press, 2002;684-704.
2. Levin H. The retrotransposons of *Schizosaccharomyces pombe*. In: Egal R, ed. The molecular biology of *Schizosaccharomyces pombe*. Heidelberg: Springer, 2002;in press.
3. Singleton T, Levin H. An LTR-retrotransposon of the fission yeast has a unique preference for a specific chromosome. Eukaryot Cell. 2002;1:44-55.

## COLLABORATORS

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# MECHANISM AND REGULATION OF EUKARYOTIC PROTEIN SYNTHESIS

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We are studying the mechanism and regulation of eukaryotic protein synthesis. An overarching interest is the universally conserved translation initiation factor eIF5B, an ortholog of bacterial translation initiation factor IF2. We have shown that eIF5B promotes ribosomal subunit joining in the second GTP-dependent step of the translation initiation pathway. In addition, we obtained the X-ray structure of eIF5B and are studying the structure/function properties of this factor and its interaction with the ribosome. We are also analyzing how the function of the factor eIF2, which binds $tRNA_i^{Met}$ to the ribosome, is down-regulated by phosphorylation. Our studies focus on the determinants of substrate recognition by the eIF2α protein kinases and on the structure and regulation of these enzymes. The human eIF2α kinase PKR is an important component of the interferon-mediated antiviral response; consequently, many viruses encode inhibitors of PKR. We have reconstituted in yeast cells the antagonism of PKR by pseudosubstrate inhibitors encoded by poxviruses. By identifying mutations in these inhibitory proteins, and others in PKR, that increase or decrease inhibition, we are identifying molecular determinants of substrate recognition in both eIF2α and PKR.

## Molecular, Biochemical, and Structural Analysis of eIF5B/IF2, a Universally Conserved Translation Initiation Factor

*Arefin, Cao, Kim, Shin, Dever; in collaboration with Burley, Pestova, Lorsch, Hellen*

We previously discovered the translation initiation factor eIF5B and characterized the factor from both yeast (encoded by the *FUN12* gene) and humans. The eIF5B is an ortholog of the bacterial translation initiation factor IF2. Working in collaboration with Tatyana Pestova and Christopher Hellen at SUNY-Brooklyn, we found that human eIF5B promotes the subunit-joining step of translation initiation. The eIF5B resembles the bacterial translation initiation factor IF2. Like IF2, eIF5B is a GTP-binding protein and possesses ribosome-dependent GTPase activity. In collaboration with Stephen Burley at The Rockefeller University, we determined, by analyzing X-ray structures, three states of eIF5B from the archaeon *Methanobacterium thermoautotrophicum*: inactive (eIF5B•GDP), active [eIF5B•GDPNP (mimicking GTP)], and nucleotide-free. The extended protein is composed of four domains and resembles a chalice. Comparison of the structures of the active and inactive forms of eIF5B revealed that modest structural changes in the GTP-binding domain (in the cup of the chalice) are amplified by an articulated lever mechanism that results in significant movement of domain IV (the base of the chalice). The GTP-binding domain and domain II of eIF5B resemble the first two domains of the translation elongation factors EF-Tu and EF-G. Based on this structural conservation, we proposed that eIF5B binds near the GTPase-activating center of the ribosome, as has been shown for EF-Tu and EF-G (Roll-Mecak *et al.*, 2000; Dever *et al.*, 2001).

The identification of eIF5B was particularly intriguing because it had been believed that only a single GTP molecule was used during translation initiation. However, the translation initiation factor eIF2, which binds the initiator Met-tRNA to the small ribosomal subunit in the first step of the translation initiation pathway, is also a GTPase. Therefore, the discovery of eIF5B predicts that two GTP molecules will be consumed during translation initiation. To test this prediction, a mutant form of human eIF5B was generated that changed the nucleotide specificity of the factor from GTP to XTP. Ribosome-dependent nucleotide hydrolysis assays confirmed that the mutation eliminated GTPase activity but endowed the factor with XTPase activity. Consistent with this altered nucleotide specificity, the mutant protein catalyzed subunit joining in the presence of XTP but not GTP. These results demonstrate that two GTP molecules are required in eukaryotic translation initiation. We propose that the GTP used by eIF2 plays a role in proper selection of the AUG start codon, whereas the GTP consumed by eIF5B promotes ribosomal subunit joining.

To assess the role of GTP-binding and hydrolysis by eIF5B, we mutated the Switch 1 motif in the factor's GTP-binding domain. Substitution by alanine of a threonine that is conserved in all GTP-binding proteins eliminated the eIF5B GTPase and translational stimulatory activities. In addition, expression of this mutant protein in yeast severely impaired cell growth. Interestingly, the mutant factor retained the ability to promote subunit joining. Thus, GTP hydrolysis by eIF5B is necessary for protein synthesis but not for subunit joining. We screened for intragenic suppressor mutations that restored the ability of the eIF5B Switch 1 mutant to promote yeast cell growth. One suppressor mutation mapped to the GTP-binding domain; however, the mutation did not restore the GTPase activity of the factor. Biochemical studies demonstrated that the suppressor mutation lowered the ribosome-binding affinity of eIF5B. Thus, we propose that GTP-binding and hydrolysis by eIF5B regulates the binding of the factor to the ribosome.

On most mRNAs, ribosomes initiate translation at the AUG codon closest to the 5' end. However, in yeast lacking eIF5B or in eIF5B mutants that lacked GTPase activity, the level of ribosomal leaky scanning increased, i.e., the ribosomes bypassed the first AUG codon and instead initiated translation at a downstream AUG codon. We propose that the GTPase activity of eIF5B serves as a checkpoint to ensure efficient subunit joining during translation initiation. Thus, when a scanning 40S ribosomal complex reaches an AUG codon, GTP hydrolysis by eIF2 results in release of initiation factors. We propose that eIF5B•GTP binds to this complex, stabilizes Met-tRNA binding, and promotes joining of the large ribosomal subunit. Upon 60S joining, eIF5B hydrolyzes GTP, and the factor is released, enabling the 80S ribosome to begin translation elongation.

### Poxvirus Pseudosubstrate Inhibitors of eIF2α Kinases: Molecular Implications for Kinase-Substrate Interactions

*Liu, Cao, Dever; in collaboration with Ramelot, Kennedy*

Previously, we demonstrated that the vaccinia virus K3L protein and the swinepox virus C8L protein are pseudosubstrate inhibitors of PKR, the mammalian interferon-induced eIF2α kinase. Expression of either the K3L or C8L protein reduced eIF2α phosphorylation and blocked the toxic effects associated with expression of PKR in yeast. This inhibition of PKR by the K3L and C8L proteins was dependent on a sequence motif KGYID, which is conserved among all K3L homologs and eIF2α and located around 30 residues C-terminal of the Ser-51 phosphorylation site in eIF2α. As part of a structural genomics initiative, Theresa Ramelot, a postdoctoral fellow in Michael Kennedy's laboratory, obtained by NMR the structure of the K3L homolog from myxoma virus, the M156R protein. It is interesting that the critical KGYID sequence motif and the region corresponding to Ser-51 in eIF2α are located on the same face of the M156R protein, likely forming the PKR recognition surface. Working in collaboration with the Kennedy laboratory, we demonstrated that the M156R protein is an efficient *in vitro* substrate for phosphorylation by PKR. Mutational analyses indicated that the primary site of phosphorylation is a Tyr residue located in the position corresponding to Ser-51 in eIF2α. In addition, the M156R protein competed with eIF2α for phosphorylation by PKR. Thus, rather than functioning as a pseudosubstrate inhibitor like the K3L protein, the M156R protein may interfere with eIF2α phosphorylation through simple substrate competition (Ramelot *et al.*, 2002).

To extend this project, we are characterizing K3L mutants that are more potent inhibitors of PKR. Whereas wild-type K3L partially suppressed the toxic effects of PKR in yeast, these hyperactive K3L proteins more fully suppress PKR toxicity in yeast. In addition, we identified PKR mutants that are resistant to inhibition by K3L while retaining the ability to phosphorylate eIF2a. By sequencing these PKR mutants, we identified 12 single amino acid changes that make PKR resistant to K3L inhibition. An interesting finding is that these mutations cluster in the C-terminal lobe of the kinase domain; based on structural modeling, they likely alter points of contact between PKR and its pseudosubstrate (or substrate). Currently, we are using genetic and biochemical assays to determine how the mutations in PKR render the kinase less sensitive to pseudosubstrate inhibition.

### Substrate Recognition by the eIF2α Protein Kinases

*Dey, Liu, Cao, Dever*

To gain insights into the mechanism of substrate recognition by the eIF2a kinases GCN2 and PKR, we are defining the minimal eIF2a substrate that is efficiently phosphorylated. We expressed and tested deletion mutants of eIF2a both *in vivo* and *in vitro* for phospho-rylation on Ser-51. Residues 1-180 of eIF2a appear to be necessary for high-level phosphorylation. Deletion of as few as 10 amino- or carboxyl-terminal residues from the eIF2a 1-180 protein severely impaired phosphorylation of Ser-51. These results, combined with the results of our studies on the K3L protein, suggest that GCN2 and PKR recognize a large domain of eIF2a. In addition, our results demonstrate that kinase-substrate recognition for eIF2a phosphorylation is not limited to a short consensus sequence element flanking the phosphorylation site, as has been demonstrated for other kinases. We employed an *in vivo* screen to identify the residues in yeast eIF2a that are critical for translational regulation and phosphorylation of Ser-51 by the eIF2a kinases. Few substitutions in the residues flanking Ser-51 affect substrate recognition; however, many mutations at these residues alter translational regulation by the eIF2a kinases. In contrast, mutations at residues 79–83, which include the KGYID sequence element that is conserved among the K3L homologs and eIF2a, significantly impaired phosphorylation of Ser-51. Of particular note is that substitution of Ala for Asp-83 at 32 residues from the phosphorylation site (at Ser-51) completely blocked eIF2a phosphorylation by GCN2 and PKR both *in vivo* and *in vitro*. Such a critical requirement for specific residues in a substrate remote from the site of phosphorylation has not been reported for any other protein kinase. The unprecedented importance of remote sequences, combined with the ease of conducting *in vivo* analyses of kinase and substrate mutants, makes the yeast eIF2a system ideal for studying substrate recognition by protein kinase.

## SELECTED PUBLICATIONS

1. Dever TE. Gene-specific regulation by general translation factors. Cell. 2002;108:545-556.

2. Dever TE, Roll-Mecak A, Choi SK, Lee JH, Cao C, Shin B-S, Burley SK. The universal translation initiation factor IF2/eIF5B. Cold Spring Harb Symp Quant Biol. 2001;66:417-424.

3. Goossens A, Dever TE, Pascual-Ahuir A, Serrano R. The protein kinase Gcn2p mediates sodium toxicity in yeast. J Biol Chem. 2001;276:30753-30760.

4. Ramelot TA, Cort JR, Yee AA, Liu F, Goshe MB, Edwards AM, Smith RD, Arrowsmith CH, Dever TE, Kennedy MA. Myxoma virus immunomodulatory protein M156R is a structural mimic of eukaryotic translation initiation factor eIF2$\alpha$. J Mol Biol. 2002;322:943-954.
5. Roll-Mecak A, Shin BY, Dever TE, Burley SK. Engaging the ribosome: universal IFs of translation. Trends Biochem Sci. 2001;26:705-709.
6. Searfoss A, Dever TE, Wickner R. Linking the 3' poly(A) tail to the subunit joining step of translation initiation: relations of Pab1p, eIF5B (Fun12p) and Ski2p-Slh1p. Mol Cell Biol. 2001;21:4900-4908.
7. Zhan K, Vattem KM, Bauer BN, Dever TE, Chen J-J, Wek RC. Phosphorylation of eukaryotic initiation factor-2 (eIF2) by HRI-related protein kinases in *Schizosaccharomyces pombe* is important for resistance to environmental stresses. Mol Cell Biol. 2002;22:7134-7146..

## COLLABORATORS

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# MOLECULAR MECHANISM OF FROG METAMORPHOSIS

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This laboratory is exploring molecular mechanisms underlying amphibian metamorphosis. The control of this developmental process by thyroid hormone (TH) offers a unique paradigm in which to study gene function in postembryonic organ development. During metamorphosis, various organs undergo vastly different changes. Some, like the tail, undergo complete resorption, while others, such as the limb, are developed *de novo*. Most of the larval organs persist through metamorphosis but are dramatically remodeled to function in a frog. For example, the tadpole intestine in *Xenopus laevis* is a simple tubular structure consisting mainly of a single layer of primary epithelial cells. During metamorphosis, a process of specific cell death and selective cell proliferation and differentiation transforms the intestine into a multiply folded adult epithelium with elaborate connective tissue and muscles. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis, both *in vivo* by using transgenesis or hormone treatment of whole animals, and *in vitro* in organ cultures, offer excellent opportunities both to study the developmental function of thyroid hormone receptors (TRs) and the underlying mechanisms *in vivo* and to identify and functionally characterize genes that are critical for postembryonic organ development in vertebrates.

## Function of TR during Development

*Buchholz, Hsia, Sachs,[a] Shi*

We have proposed a dual function model of TR action based on our earlier studies in the oocyte, developing embryos, and tadpoles. The model postulates that heterodimers between TR and RXR (9-*cis* retinoic acid receptor) activate gene expression during metamorphosis when TH is present while, in premetamorphic tadpoles, they repress gene expression to prevent metamorphosis, thus ensuring a tadpole growth period. Our studies have since provided strong support and mechanistic insights for such a model. Most recently, we have shown that the corepressors N-CoR and SMRT are recruited to TH-response genes in premetamorphic tadpoles and are released upon treatment of the tadpoles with TH, indicating that unliganded TR recruits these corepressors to repress target genes in tadpoles. In agreement with the observation that these corepressors can form complexes containing histone deacetylases (HDACs), TH treatment leads to a rise in local histone acetylation at the TH response genes, at least in the intestine and tail, arguing that histone acetylation is an important factor in gene regulation by TR.

To investigate the function of TR *in vivo*, we have adapted a sperm-mediated transgenic method to generate transgenic animals expressing a dominant-negative TR. Phenotypic analyses indicate that over-expression of the dominant-negative TR inhibits TH-induced metamorphosis. More important, we have shown that the dominant-negative TR specifically blocks the expression of TH response genes that we and others have already identified. These results provide molecular evidence for the central role of TR in regulating the response genes that mediate the effects of TH on metamorphosis.

### Roles of Cofactors in Gene Regulation by TR

*Amano, Jones,[b] Obata, Paul, Tomita, Wade[c], Shi*

TR regulates gene transcription by recruiting cofactors to target genes. In view of the several biochemical and molecular studies already completed on such cofactors, we are investigating how TR utilizes different cofactors in the context of development in various organs. We have recently characterized one coactivator, the *Xenopus* TRIP7. However, our studies using the frog oocyte model system suggest that *Xenopus* TRIP7 has a relatively small effect on TR function, and thus its role in developmental gene regulation by TR may be limited. Accordingly, we have begun to study p300 and SRC, evidence for whose involvement in TR function has been obtained from *in vitro* and tissue culture cell studies. We have obtained the cDNA clones for *Xenopus* p300, SRC1, and SRC2 and have shown that they are expressed during metamorphosis.

With regard to the corepressors mentioned above, we have shown that the corepressors N-CoR and SMRT are recruited to TH response genes in developing tadpoles and that histone acetylation is involved in gene regulation by TR in tadpoles. Both N-CoR and SMRT are known to interact with other proteins. To investigate how these corepressors participate in the repression by unliganded TR, we have isolated three N-CoR-corepressor complexes. However, due to low abundance, we have yet to determine the identities of the components in the complexes. On the other hand, in HeLa cells, the TBL1 (transducing-b-like 1) protein has been shown to exist in an N-CoR complex that was identified by others. We have cloned the frog homolog and intend to investigate whether TBL1 is present in our N-CoR complexes and whether it participates in N-CoR-dependent gene repression by TR. We intend to use chromatin immunoprecipitation assays to determine the recruitment of both corepressors and coactivators by TR to target genes in tadpoles. To investigate directly the function of these factors *in vivo*, we will precociously over-express wild-type or dominant-negative cofactors and analyze the resulting effects on animal development and target gene expression (and local changes in chromatin structure, when appropriate). Before undertaking transgenic studies, we will characterize the cofactors/mutants for their ability to influence TR function in the frog oocyte system that we established earlier for studying gene function in the context of chromatin. This approach should help in the rational design of our transgenic studies on the molecular mechanisms responsible for the effects of the cofactors.

### Involvement of Matrix Metalloproteinases during TH-Induced Tissue Remodeling

*Amano, Damjanovski,[d] Fu, Wei, Shi; in collaboration with Ishizuya-Oka*

In an effort to identify genes important for post-embryonic development, we have isolated many TH response genes during metamorphosis. Expression analyses and other studies have led us to focus on the TH-response genes encoding matrix metalloproteinases (MMPs) for functional investigations. MMPs are extracellular enzymes capable of digesting various extracellular matrix (ECM) components. Our earlier studies led us to propose that the MMP stomelysin-3 (ST3) is directly or indirectly involved in ECM remodeling, which in turn influences cell behavior. By using a function-blocking antibody against the catalytic domain of ST3, we have demonstrated in organ cultures that blocking ST3 function inhibits TH-induced apoptosis of larval intestinal epithelial cells as well as the invasion of the proliferating adult epithelial cells into the connective tissue. These effects are accompanied by an inhibition of the remodeling of the basal lamina or basement membrane, the ECM that separates the connective tissue from the epithelium. The results support the argument that ST3 is directly or indirectly involved in ECM remodeling, which in turn influences cell behavior. To investigate directly the roles of MMPs in developing animals, we are employing the transgenic approach to express wild-type and mutant MMPs in *Xenopus* embryos and tadpoles. In our initial study, we over-expressed *Xenopus* MMPs stromelysin-3 (ST3) and collagenases-4 (Col4) under the control of a ubiquitous promoter and observed that embryos with over-expressed ST3 or Col4, but not the control green fluorescent protein (GFP) or a mutant ST3, died in a dose-dependent manner during late embryogenesis. This lethality in early development prevented us from investigating the roles of MMPs during metamorphosis. Thus, we have developed a double-promoter approach described below to over-express MMPs under an inducible promoter. In our preliminary study, we generated transgenic tadpoles by using a double-promoter construct in which ST-3 is under the control of the heat shock–inducible promoter. Heat shock at tadpole stages led to over-expression of stromelysin-3 in all organs, although no visible morphological changes of the tadpoles were observed for four days. Analysis of the intestine showed that over-expression of stromelysin-3 caused premature apoptosis in the tadpole epithelium, consistent with our earlier organ culture studies. We are currently investigating how the transgenic animals undergo natural and TH-induced metamorphosis.

### Development of a Double-Promoter Transgenic Approach

*Buchholz, Fu, Shi*

In our first set of transgenic experiments, we over-expressed three different MMPs under the control of the ubiquitous CMV promoter. We found that all the animals expressing the wild-type MMPs, but not a catalytically inactive stromelysin-3 or GFP, died at various stages of development, none developing into metamorphosing tadpoles. These initial studies identified two problems with the existing approach. The first is embryonic lethality caused by MMP expression under a ubiquitous promoter, and the second is the difficulty in identifying transgenic animals. Normally, transgenic animals or plants are identified by PCR typing, which is tedious and slow. Alternative approaches include the use of GFP-fusion proteins and cotransgenesis involving two plasmids, one expressing the desired gene and the other a marker gene. The first approach is limited by the possibility that GFP may not be visible if the transgene is not expressed on the surface of the animal or if the fusion protein has reduced fluorescence or is expressed only at certain developmental stages. The second is also of limited use because of the relatively low efficiency of chromosomal integration of both plasmids in a single transgenic animal.

To overcome these problems, we have developed a double-promoter approach. In the construct for transgenesis, we use a lens-specific promoter ($\gamma$-crystalline promoter) to drive the expression of GFP and a second, heat shock–inducible promoter (or another promoter of interest) to drive the expression of the desired gene. This approach allows us to generate transgenic animals that can be easily identified by checking the eyes under UV and that can be maintained and treated in the same containers as nontransgenic controls. We have validated the approach by using PCR typing of transgenic animals and demonstrating that both promoters function independently and properly as designed.

## SELECTED PUBLICATIONS

1. Amano T, Leu K, Yoshizato K, Shi YB. Thyroid hormone regulation of a transcriptional coactivator in *Xenopus laevis*: implication for a role in postembryonic tissue remodeling. Dev Dyn. 2002; 223:526-535.
2. Damjanovski S, Amano T, Li Q, Pei D, Shi YB. Overexpression of matrix metalloproteinases leads to lethality in transgenic Xenopus laevis: implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev Dyn. 2001; 221:37-47.
3. Damjanovski S, Sachs LM, Shi YB. Function of thyroid hormone receptors during amphibian development. In: Methods of molecular medicine: thyroid hormone receptors. Totowa, NJ: Humana Press, Inc., 2001;202:153-176.
4. Fu L, Buchholz D, Shi YB. A novel double promoter approach for identification of transgenic animals: a tool for *in vivo* analysis of gene function and development of gene-based therapies. Mol Reprod Dev. 2002;62:470-476.

5. Hsia SC, Shi YB. Chromatin disruption and histone acetylation in the regulation of HIV-LTR by thyroid hormone receptor. Mol Cell Biol. 2002;22:4043-4052.
6. Ishizuya-Oka A, Amano T, Fu L, Shi YB. Regulation of apoptosis by extracellular matrix during postembryonic development in *Xenopus laevis*. In: Lockshin RA, Zakeri Z, eds. When cells die II: a comprehensive evaluation of apoptosis and programmed cell death. New York: John Wiley & Sons, 2002;in press.
7. Jones PL, Sachs LM, Rouse N, Wade PA, Shi YB. Multiple N-CoR complexes contain distinct histone deacetylases. J Biol Chem. 2001;276:8807-8811.
8. Jones PL, Shi YB. N-CoR-HDAC corepressor complexes: roles in transcriptional regulation by nuclear receptors. In: Workman JL, ed. Current topics in microbiology and immunology: protein complexes that modify chromatin. Berlin: Springer-Verlag, 2002;in press.
9. Sachs LM, Amano T, Rouse N, Shi YB. Involvement of histone deacetylase at two distinct steps in gene regulation during intestinal development in Xenopus laevis. Dev Dyn. 2001;222:280-291.

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# CONTROL OF CHROMOSOME TRANSMISSION FIDELITY BY THE SMC-BASED PROTEIN COMPLEXES

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The eukaryotic ATP-ases of the SMC (structural maintenance of chromosomes) family are located in the core of two essential eukaryotic protein complexes: cohesin and condensin, which determine the higher-order chromosome structure in proliferating cells. The condensin complex is the chief molecular machine of chromosome condensation and is indispensable for proper separation of chromatids during anaphase. It is also likely to be involved in formation of the higher-order structure of interphase chromosomes. Cohesin is a four-subunit complex essential for establishment and maintenance of a physical association between sister chromatids (sister chromatid cohesion, SCC); it is also implicated in a number of interphase chromatin-mediated processes: control of gene expression, DNA replication, and DNA repair. To understand the molecular mechanisms of the SMC-containing complexes, we combined genetic, cytological, and biochemical approaches. To investigate the mechanisms determining the specificity of condensin's targeting to the natural chromatin sites, we set up a genetic screen to isolate mutants that are defective in mitotic targeting of condensin to chromatin and investigated the input of the Smt3-modification pathway in condensin regulation. Studies on eukaryotic cohesin were complemented by collaborative work on bacterial SMC proteins that resulted in characterization of a four-subunit SMC complex from bacilli.

## Condensin Regulation and Chromatin Targeting

*Wang, Yong-Gonzales, Lee*

Condensin is a five-subunit, evolutionarily conserved protein complex indispensable for the process of chromosome condensation in mitosis and meiosis. Despite substantial recent progress in understanding the molecular structure and enzymology of condensin, mechanisms regulating its activity remain obscure. Our studies on condensin focus on the budding yeast complex composed of five essential subunits: Smc2, Smc4, Ycs5/Ycg1, Ycs4, and Brn1. We designed a genetic screen to isolate and identify new mutants that are defective in mitotic targeting of condensin to rDNA. The genetic studies established that at least two new pathways control condensin localization to the condensing chromatin domains in mitosis and thus the chromosome condensation process. One pathway requires proper establishment of sister chromatid cohesion, as both cohesin mutations and the absence of sister chromatids (due to bypass of DNA replication) impair condensin targeting as well as chromosome condensation. The second pathway is the branch of the SUMO-modification pathway. We established that SUMO (Smt3) itself, Smt3-isopeptidase Smt4, and the E3 Smt3-ligases Siz1 and Siz2 play a crucial role in condensin targeting. The molecular mechanism of this regulation is under investigation. Using a whole proteome approach, we initiated a search for the essential targets of Smt3 conjugation, with a special emphasis on chromatin proteins.

### Cohesin-Chromatin Interaction

*Kagansky, Lukyanov*

SCC is an essential process that establishes and maintains the link between two sister chromatids from S-phase through anaphase. SCC regulation in mitosis and meiosis is extremely complex but is mostly directed at cohesin, an evolutionarily conserved four-subunit protein complex. In budding yeast, cohesin includes essential proteins Smc1, Smc3, Scc1/Mcd1, and Scc3. The molecular mechanism of cohesin activity in SCC is largely unknown. Thus, we established a purified system to study SCC *in vitro*. It includes a model chromatin template and the purified cohesin complex. We purified the recombinant cohesin holocomplex, its Smc1-Smc3 ATP-ase core, and the non-SMC subunits Scc3 and Mcd1. Experiments with the defined chromatin probe showed that the Smc1p-Smc3p dimer and cohesin holocomplex differ dramatically in their chromatin-binding properties. Only full cohesin is able to form a stoichiometric complex with chromatin. Binding of cohesin to chromatin occurred independently of linker DNA and histone tails. Our experimental approach allowed us to gain insights into the mechanism of the elementary sister chromatid cohesion reaction.

### Discovery of a Bacterial SMC Complex

*Strunnikov; in collaboration with members of Graumann's laboratory*

SMC proteins forming the core of condensin and cohesin in eukaryotes are also ubiquitous in bacteria. Chromosome segregation is severely impaired in the SMC *Bacillus subtilis* mutants, yet the molecular role of SMC proteins in bacterial cells is unknown. It was believed that, in bacteria, SMC proteins do not complex with other proteins. However, our biochemical analysis of the SMC complex from bacilli combined with genetic and cell biology data from Dr. Graumann's group indicates that the bacterial SMC complex does include four subunits: the SMC dimer and two novel proteins ScpA and ScpB. Analysis of the SMC complex composition, localization, and function suggests that it is reminiscent of cohesin and probably drives packaging of bacterial chromatin *in vivo*.

## SELECTED PUBLICATIONS

1. Mascarenhas J, Soppa J, Strunnikov AV, Graumann PL. Cell cycle-dependent localization of two novel prokaryotic chromosome segregation and condensation proteins in Bacillus subtilis that interact with SMC protein. EMBO J. 2002;21:3108-3118.
2. Meluh PB, Strunnikov AV. Beyond the ABCs of CKC and SCC. Do centromeres orchestrate sister chromatid cohesion or vice versa? Eur J Biochem. 2002;269:2300-2314.
3. Strunnikov AV, Aravind L, Koonin EV. Saccharomyces cerevisiae SMT4 encodes an evolutionarily conserved protease with a role in chromosome condensation regulation. Genetics. 2001;158:95-107.

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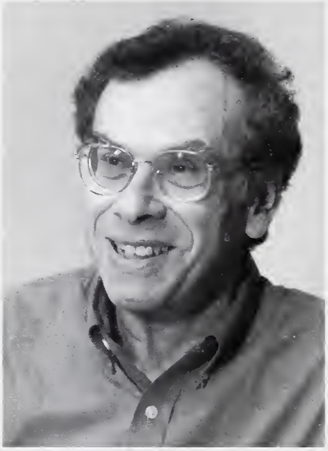

We study various elements of cell processes involved in signal transduction, protein trafficking, and cell motility. Our focus is on understanding the interactions and transformations of supramolecular cellular assemblages such as protein-coated endocytic vesicles, metabolic signalling networks, and extended cytoskeletal structures. We are particularly interested in the way complex activities are coordinated in space and time and thus devote considerable effort to developing and applying novel methodologies based on mathematical and physical principles, most notably the construction of specialized fluorescence-based optical instrumentation and the use of advanced electromagnetic scattering techniques that allow examination of structures and dynamics on nanoscopic-length scales. We also develop mathematical models of specific aspects of supramolecular and cellular behavior.

### Mechanics of Coated Vesicle Formation

*Nossal, Boukari, Muthukumar*

Much protein and lipid trafficking in eukaryotic cells involves small tubulo-vesicular entities whose generation from cell membranes is mediated by the binding of specific coat proteins. We are attempting to understand the physical mechanisms by which such structures are generated. In earlier studies, we developed methods to determine the mechanical properties of clathrin cages (the major structural elements of the protein coats of certain endocytic vesicles) and examined how the binding of accessory proteins can increase the rigidity and stability of the cages. Recently, to investigate a possible relationship between total clathrin concentration and the amount of polymerized product, we adapted an analytical model of clathrin cage assembly that accounts for the combined bending and flexing rigidities of triskelion legs and hubs, the intrinsic curvature of an isolated triskelion, and free energy changes associated with interactions between legs of neighboring triskelions. The analysis, which is similar to that used to study micelle formation by amphiphilic molecules, provides insight into the ways that intra- and intermolecular forces determine the critical concentration for polymerization (below which cages will not form) and the size distributions of the resultant structures. We found that the critical concentration can decrease below a detectable level when the interactions between the legs of neighboring triskelions in a cage are sufficiently strong. We determined that, in general, the greater the free energy change associated with cage formation (i.e., the more stable the cages), the lower is the critical concentration. We have also determined how the critical concentration explicitly depends on the various system variables (rigidity and out-of-plane curvature of clathrin triskelions and inter-leg association energies) and have begun to explore ways that such inferences may be tested by fluorescence correlation spectroscopy (FCS) and other physical techniques that might be used to measure sizes and shapes of supramolecular assemblies.

### Mathematical Analysis of Cyclic Metabolic Pathways for Phosphoinositide Synthesis

*Skupsky, Nossal*

Considerable evidence indicates that phosphatidylinositide (PI) metabolism is involved in many cell-physiological events, including vesicle biogenesis, motility, and chemotaxis. The feedback and feed-forward character of cyclic pathways of PI metabolism suggests that PIs may act as biochemical switches. Several investigators have proposed a direct role for PIs as spatial regulators of endocytic vesicle production, as it has been known for some time that PI 3-kinase (PI3K) activity increases upon binding of growth factors or various other ligands to cell surface receptors, leading to conversion of phosphatidylinositol 4,5 bisphosphate ($PIP_2$) to phosphatidylinositol trisphosphate ($PIP_3$). Additional feedback loops can arise from coupling to membrane curvature. For example, PI3K activity can be enhanced if its substrate is embedded in a curved membrane while PIs may transiently induce membrane bending through their electronic charge or by attracting curvature-inducing cytosolic proteins (e.g., epsin 1). Using mathematical and computational methods, we have examined a biochemical model for regulation of PI signalling that includes the actions of PI kinases and phosphatases, phospholipase C, small g-proteins, and phosphatidic acid production. In addition to providing insight into vesicle formation in endocytosis, the model has permitted us to understand recent data implicating PI signalling in chemotaxis. In fact, a model of eukaryotic gradient sensing that is consistent with current data and based on PI cycles can be constructed in several ways. For example, our model shows qualitative differences depending on how non-linearities are included in the chemical kinetics and whether species translocating from the cytoplasm to plasma membrane exist in small or large quantities. We have begun an analysis of spatial patterns that might be triggered by an outside stimulus, depending on different values of adjustable system parameters.

### Structure of Tubulin Polymers

*Sackett, Watts, Nossal; in collaboration with Krueger, Steven, Chernomordik, Losert*

Tubulin polymers are participants in various critical cell functions, including mitosis, intracellular transport, determination of global cell morphology, and cell motility. Polymers of purified tubulin take many forms in response to environmental factors such as pH, salt concentration, temperature, and the presence of small organic molecules that mediate tubulin-tubulin interactions. We have been investigating how several of these factors affect supramolecular tubulin structure. Specifically, we have used small-angle neutron scattering (SANS) to examine, for example, taxol-stabilized microtubules and other tubulin samples in both $H_2O$ and $D_2O$ buffers. We made measurements at pH/pD values between 6.0 and 7.8, with observed scattered intensities, $I(Q)$, interpreted in terms of multi-component models of microtubules and related tubulin polymers. A semi-quantitative curve-fitting procedure was developed to estimate the relative amounts of the supramolecular components of the samples. We were able to show that, when pD is lowered, the samples appear to contain an appreciable amount of sheet-like structures in addition to microtubules and that the average microtubule protofilament number increases from ca. 12.5 at pD 7.0 to ca. 14 at pD 6.0. These results are of considerable interest, as several studies have demonstrated that high concentrations of $D_2O$ can be cytotoxic to mammalian cells, presumably due to inhibition of mitosis. We now have insight into the molecular changes that are involved. Moreover, extensions of our work may lead to a deeper understanding of the chemical physics generally underlying kinetically driven supramolecular assembly processes.

In a different study, we examined the mechanisms whereby cryptophycin 1 (a potential anti-cancer drug) interacts with tubulin to induce the formation of ring-like complexes. By combining results of sedimentation velocity and dynamic light scattering measurements with scanning transmission electron microscopy measurements, we obtained a ring mass that, in combination with cryoelectron microscopy, gives an accurate representation of ring structure. We thereby were able to infer that two well-defined, repeating bends create the necessary curvature to cause the ring structures to form. We are planning extensions of these investigations to understand why microtubules self-organize to form large-scale arrays; the latter are amenable to study by optical methods such as polarization and confocal microscopy, optical birefringence, and, possibly, laser tweezers.

### Fluorescence Correlation Spectroscopy and Fourier Imaging (Scattering) Techniques

*Boukari, Nossal, Sackett; in collaboration with Szu*

We recently built a microscope-based, dual-channel fluorescence correlation spectrometer that allows us to determine the translational diffusion coefficients (and therefore the sizes) of macromolecular complexes when in solution at very low concentrations. We have used the device to characterize the unusual polymers resulting from the interaction of tubulin with cryptophycin, hemiasterlin, and dolastatin; the last is among a group of antimitotic marine natural products undergoing evaluation as potential anti-tumor drugs. These peptides inhibit tubulin polymerization into microtubules and instead induce the formation of single-walled tubulin rings of 27-nm diameter in the case of cryptophycin and 44-nm diameter in the case of hemiasterlin and dolastatin, as revealed by electron microscopy of drug-tubulin samples of relatively high (micromolar) concentration. However, by using FCS to monitor the hydrodynamic diameter and the apparent number of fluorescent entities as the samples are diluted, we have determined that cryptophycin-tubulin rings are stable even with tubulin concentrations as low as 1 nM, whereas hemiasterlin-tubulin rings depolymerize even at relatively high concentrations (100 nM). Dolastatin-tubulin rings demonstrate an intermediate level of stability. Comparison of cytotoxicity measurements taken on several cell lines shows a rough correlation between that of the drugs and the stability of the rings.

These studies, which are interesting in their own right, also serve as prototypes for applications involving different biomacromolecular systems. Thus, we have initiated studies of the structure and stability of protein-polysaccharide conjugates that are currently under development as vaccines against enteric pathogens. Our objective is to determine the sizes of vaccine conjugates when diluted to low physiological concentrations in a range not easily accessible to study by other physical techniques. We also plan to use the methodology to examine the assembly of reconstituted clathrin cages as well as parameters pertaining to interactions of cellular organelles. In a related investigation, we have used SANS to examine a variety of tubulin polymers in solution, including isolated rings and higher-order ring aggregates that appear when dolastatin acts on tubulin. Preliminary evidence indicates that the aggregates are composed of columnar stacks of elemental, individual rings.

## SELECTED PUBLICATIONS

1. Nossal R. Energetics of clathrin basket assembly. Traffic. 2001;2:138-147.
2. Nossal R, Zimmerberg J. Endocytosis: curvature to the ENTH degree. Curr Biol. 2002;12:R770-R772.
3. Watts NR, Cheng N, West W, Steven AC, Sackett DL. Cryptophycin-tubulin ring structure indicates two points of curvature in the tubulin dimer. Biochemistry. 2002;41:12662-12669.

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We develop new biophysical and optical methodologies for biomedical research and clinical applications. Of late, we have been focusing on the refinement of technological approaches to characterizing early stages of disease and to developing strategies for prevention of progression and for monitoring responses to therapy in cancer and age-related macular degeneration (AMD). In further developments of Laser Capture Microdissection and related laser technologies, we have coupled the isolation of small populations of cells from sections of complex tissues with global analysis of gene expression. We have applied statistical analysis of gene expression to identify candidate lists for stage-specific molecular markers within microdissected cell populations, focusing on improvements necessary to identify critical but less abundantly expressed genes.

### Laser Microdissection Technology Development

*Mertts, Obiakor, Parlato, Bonner; in collaboration with Mage, Obiakor, Emmert-Buck*

As the list of expressed human genes is completed, a major scientific and medical challenge will be to track the complex molecular events that drive normal tissue differentiation and progression of pathologic lesions. With refinements in PCR, hybridization microarrays, and mutation screening, both DNA and mRNA can be extracted from tissue biopsies or cytology specimens and analyzed with a parallel panel of hundreds or even thousands of genetic markers. Given that cells in complex tissue are biochemically and physically affected by surrounding cells as well as by remote stimuli from greater distances, the task of analyzing critical gene expression patterns in development, normal function, and disease progression depends on the extraction of specific cells from their complex tissue milieu. Our laboratory, in collaboration with NCI, has invented and developed Laser Capture Microdissection (LCM) (Arcturus Engineering, Inc., has commercialized the technology). LCM provides a rapid, reliable method to procure pure populations of specified cells from microscopic regions of tissue sections in a form useful for subsequent quantitative, multiplex molecular analysis. Routine clinical use of this approach will require the reliable preservation of the macromolecules of interest within a sample, assurance that critical clonal populations are included and can be identified within an *ex vivo* sample, and quantitative extraction and analysis of critical subsets of marker macromolecules.

In an effort to lead technology integration with biological and clinical research, we are studying the polymer physics and chemistry of the LCM processes and competitive ablative laser microdissection techniques. Recent improvements include "noncontact LCM" prototypes that use automated beam steering and pulse sequences. The prototypes allow reliable activation of flexible transfer films and thus accommodate the injection of picoliter volumes of polymer into individually targeted cells, permitting capture and retraction of the cells over precisely controlled air gaps of 10 to 20 microns. A new method for simultaneous complete release of targeted cells from the microscope slide is based on simultaneous ablation of 100-nm-thick polymer coatings. Using standard aqueous tissue extraction buffers, we have demonstrated quantitative recovery of DNA, mRNA, and proteins from the injected polymer matrix. The LCM process, which loads picoliter cell volumes onto equivalent volumes of polymer matrix, is being applied to integrated purification and analysis of specific macromolecular constituents. We have been able to emboss a patterned microfluidic circuit into the LCM polymer tapes and directly load selected cells into a specified well of approximately one nanoliter volume.

In parallel efforts, we have been investigating the biophysics of UV laser ablation and have demonstrated the role played by polymer breakdown and ionization in electrostatic interactions determining target collection efficiency. Polymer multilayer coatings with charge neutralization appear to improve ablative laser microdissection, which has particular advantages for collecting larger populations of cells required for automated cytometry and many proteomics discovery efforts. In collaboration with NIAID, we have used nested PCR and DNA sequencing to demonstrate reliable molecular analysis in LCM-isolated immunolabeled single cells at the single-molecule level. As we proceed to single-cell isolation and greater knowledge of molecular changes in pathology, specific molecular targeting will become increasingly important to ensure rapid, precise collection of subpopulations that are not discernible by morphology alone. We are incorporating fluorescence microscopy and real-time image analysis methods, including remote image–guided sample targeting.

### Gene Expression Complexity and Statistics of Gene Expression

*Kuznetsov, Stepanov, Bonner; in collaboration with Emmert-Buck, Mulshine*

If microdissection and molecular analysis can be made clinically practical, the expression levels of sets of approximately 20 to 100 critical, stage-specific disease markers within a selected cell population might provide reliable diagnosis and intermediate endpoints of response to molecular therapies in individual patients. We have been developing robust statistical tools to facilitate identification of critical genes and pathways that characterize both normal and aberrant cellular function, particularly those suitable for "multiplex" analysis of a small number of specific cells. Analysis of large gene expression databases (SAGE and LCM cDNA libraries) suggests that a large fraction of all genes is expressed in any specific cell type and that the genes' expression levels universally exhibit a highly skewed power-law distribution similar to those characterizing many other complex systems. We are developing mathematical models for the evolution of such distributions that evidence increasing biological complexity.

To follow macromolecular changes during cancer progression within prostate, breast, lung, and ovary tissues, we have developed, in collaboration with NCI, standard procedures for the isolation of normal and pathological cells from clinical specimens. Using statistical multivariate analysis, we have developed mathematical algorithms for the selection of "candidate genes" from cDNA libraries. The candidate genes' expression frequencies have a strong correlation with disease progression. We use our models of the statistics of expression levels in cell populations to identify genes differentially expressed in cancer progression. To date, our analysis points to a critical role for many less abundantly expressed genes at a critical stage of ovarian cancer progression and suggests that, for most cancers, critical diagnostic marker sets should include such low-abundance transcripts. This notion is guiding our research in statistics of low-level gene expression and suitable detection methods.

### Gene Expression during Normal Development and Pathology Progression

*Parlato, Malekafzali, Bonner; in collaboration with Gruber, DiLauro, Mushinski*

We have applied single-cell LCM capture technology to studies of gene expression during normal development (spermatogenesis and thyroid bud development) and in stage-specific pathological cells (e.g., cancer precursors). In collaboration with Roberto DiLauro's laboratory, we examined gene expression patterns associated with the primordial thyroid and the adjacent cells from which it derives in both wild-type and knock-out mice. In collaboration with Fred Mushinski, we are applying the same LCM methodology to study early changes in the well-established plasmacytoma model in BALB/c mice. Jointly, we have developed robust LCM techniques for isolation of specific cells from mouse cryosections and recovered good yields of high-quality mRNA. In collaboration with Life Technologies, Inc., we have made cDNA libraries of high diversity and message length from 100 ng of microdissected mRNA (~5,000 cells). Using Affymetrix microarray hybridization, we can determine global gene expression profiles in these libraries while preserving the libraries for isolation of the full-length message of identified stage-specific genes. We are planning to apply these methods, which have undergone validation in reproducible animal models, to clinical specimens of early stages of disease progression in which microdissection of critical rare cell populations is required. We foresee an evolution of molecular diagnosis from one based on the qualitative or quantitative analysis of a few key macromolecules to one in which special clustering algorithms analyze complex multivariate databases. Such analyses should permit a more complete identification of highly correlated clinical cases and allow us to characterize their response to molecular therapies specifically designed to prevent progression. To this end, we have refined the LCM of rare cell populations, particularly those that might be accessible by serial, minimally invasive cell harvesting from patients. We have continued to organize and hold an annual conference at NIH on Laser Capture Microdissection and Macromolecular Analysis of Normal Development and Pathology, bringing together about 500 researchers to discuss research and methodological advances made possible by LCM.

### Prevention of Progression of Age-Related Macular Degeneration through Photoprotection

*Meyers, Ostrovsky, McConnell, Bonner*

Age-related macular degeneration (AMD) is the most common cause of severe visual loss in the elderly. Early stages of the disease are ophthalmoscopically detected with increasing frequency with age (particularly over 60 years). Lipofuscin accumulates with age in the retinal pigment epithelium (RPE). It is the dominant fluorphore in the retina, and, in Stargardt's disease, its increased rate of accumulation is associated with early macular degeneration. This finding led us to hypothesize in the early 1980s that lipofuscin is photochemically active and responsible for acute retinal damage at high blue light levels, causing chronic photochemical (free radical production) stress that increases with age (lipofuscin accumulation). If lipofuscin photochemistry plays a critical role in initiation and progression of early AMD, then simple optical filters (yellow-colored lens) might be expected to reduce dramatically the rate of photochemical free radical production. We designed such filters (Bonner: 500-nm-long pass sunglasses; Ostrovsky: 430-nm-long pass intraocular lens), which have been used clinically. The recent discovery of a photochemical active product (A2E) created by rod photoreception at high light levels and our experimental determination of A2E photo-chemical action spectrum in RPE cell culture led us to reinvestigate optimal designs for optical filters to reduce or prevent progression of early AMD. We have generated models of spectral irradiance in the human macular RPE following cataract surgery and intraocular lens (IOL) implantation and as the lens ages. The models suggest the importance of the reduction of both the maximal bleaching of rod rhodopsin and the rate of singlet oxygen generation from photo-toxic photoproducts (trans-retinal, A2E, and its photoproducts), along with the significance of the role of photodegradation of A2E and its toxic photoproducts by visible light. We have proposed new IOLs and long wavelength transmitting "sunglasses" for reducing rod activation in bright ambient light as well as the accumulation and activation of toxic byproducts of photo-transduction. In addition, we are seeking to characterize spectrally different RPE photoproducts to evaluate the potential for noninvasive monitoring of the molecular effects of such filters in patients.

## SELECTED PUBLICATIONS

1. Kuznetsov VA, Knott GD. Modeling tumor regrowth and immunotherapy. Math Comput Model. 2001;33:1275-1287.
2. Kuznetsov VA, Knott GD, Bonner RF. General statistics of stochastic process of gene expression in eukaryotic cells. Genetics. 2002;161:1321-1332.
3. Obiakor H, Sehgal D, Dasso JF, Bonner RF, Malekafzali A, Mage RG. A comparison of hydraulic and laser capture microdissection methods for collection of single B cells, PCR, and sequencing of antibody VDJ. Anal Biochem. 2002;306:55-62.
4. Parlato R, Rosica A, Cuccurullo V, Mansi L, Macchia P, Owens JD, Mushinski JF, De Felice M, Bonner RF, DiLauro R. A preservation method that allows recovery of intact RNA from tissues dissected by laser capture microdissection. Anal Biochem. 2002;300:139-145.

## PATENT

Bonner RF, Goldstein SR. Smith PD. Pohida TJ. Precision laser capture microdissection utilizing short pulse length. United States Patent #6,420,132 issued July 16, 2002.

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# APPLICATIONS OF PHOTON MIGRATION TO TISSUE TOMOGRAPHY AND SPECTROSCOPY

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Our goal is to devise new approaches for noninvasive, quantitative optical spectroscopic and tomographic imaging of deep tissue structures for clinical screening and monitoring of physiological parameters. To achieve our goal, we have undertaken a multifaceted theoretical, computational, experimental, and clinical research program that includes the time-resolved transillumination of thick tissue applied to quantitative spectroscopy of breast tumors and the use of specific fluorescent markers (e.g., ligands) for identifying molecular biology of disease processes applied to noninvasive biopsy of Sjögren's syndrome and for lymphatic imaging for sentinel node detection. We are involved in several clinical studies, including an NCI protocol to use oblique angle reflectometry for noninvasive monitoring of inflammation in oral cavity, an NIDCR/NINDS clinical study to evaluate the drug response of patients experiencing complex regional pain syndrome, and another NCI-sponsored clinical trial to evaluate the effectiveness of anti-angiogenesis drug treatment for Kaposi sarcoma.

## Time-Resolved Tomography of Thick Tissue

*Chernomordik, Hattery, Gandjbakhche; in collaboration with Zaccant, Cubbedu, Rinneberg*

In collaboration with researchers who have provided *in vivo* measurements on human breast at the Physikalisch-Technische Bundesanstalt of Berlin and Politecnico di Milano, we have developed and are using random walk methodology to quantify optical properties of breast tumors. We have performed data analysis on distributions of times of flight for photons transmitted at several wave lengths through the breast as recorded *in vivo* by time-domain scanning mammography developed by the Berlin and Milan groups. We are able to reconstruct the size of the tumors and their optical properties and those of the surrounding tissue. We have analyzed 10 sets of data and, from the absorption coefficients, estimated blood oxygen saturation and total blood volume for the tumors and the surrounding tissue. We found that some tumors, mostly invasive ductal carcinoma, are hypoxic and have increased blood volume. We are studying the correlation between the spectroscopic signatures of the breast tumors and the risk associated with the outcome of the therapy. We are continuing to refine our theoretical algorithms to make the analysis less time-consuming.

## 3D Reconstruction of Localized *in Vivo* Fluorescence

*Chernomordik, Hattery, Gannot I, Gannot G, Gandjbakhche*

The development of specific fluorescently labeled cell surface markers has opened the possibility of specific and quantitative noninvasive diagnosis of tissue changes. We are pursuing the development of a fluorescence scanning imaging system that can perform a "noninvasive optical biopsy" of Sjögren's syndrome (SS), potentially replacing the currently used histological biopsy. In our animal experiments, we have devised an imaging system to quantify the concentration of fluorescinated antibodies binding to B or T tumor cells previously injected into the oral cavity of mice. Preliminary results show that our imaging system along with our theoretical algorithm is able to monitor noninvasively the concentration of fluorophore signals as well as the pharmacokinetics associated with wash-out of the antibodies.

We are also continuing our study on the use of infrared-dependent fluorescent detection methods to determine the position of sentinel node(s) to replace currently used detection by radioactive particles. We have extended our analysis of different phantom data and *ex vivo* tissue to confirm the potential of the random walk–based theoretical approach to reconstruct with good accuracy three-dimensional positions of deeply embedded fluorophores. We are starting animal experiments with near-infrared nanoparticles to study the drainage of the particles through the lymphatics.

### Oblique Angle Reflectometry for Noninvasive Monitoring of Inflammation: Application to Chemopreventive Drugs

*Hattery, Hekmat, Gandjbakhche; in collaboration with Mulshine*

Inflammatory cell populations produce cytokines that can specifically stimulate growth of evolving cancer clones. Given that normal epithelium shares many biological properties with cancer cells, it also responds to the chronic presence of mitogenically active cytokines by accelerated growth (hyperplasia): normal cell hyperplasia provides a measure of the promotional environment of a cancer clone. At NCI, a Phase II trial is under way to determine the effectiveness of cyclooxygenase (COX) inhibitor on oral leukoplakia. The effectiveness will be monitored by surgical biopsy with its associated morbidity. As a less morbid approach to monitoring patient response, we have developed a noninvasive quantitative epithelial inflammation measuring device that may be used to evaluate the general state of the oral mucosa and monitor the effectiveness of chemopreventive treatment regimes. The device measures diffuse reflectance from a low-power optical wavelength source, which inserts photons at specific angles into the tissue. As the angle of insertion approaches parallel with the epithelial surface, the mean penetration of the photons becomes smaller and the photons spend more time in the epithelial layer. Wave lengths of interest are those with high tissue absorption, thus limiting detected photons to those with few scattering events and confining to the surface layers the tissue volume that has been interrogated. From a theoretical point of view, the approach permits the use of integral equations to describe photon migration in a two-layer tissue model. We performed several phantom experiments that used acrylamide gel phantoms with high absorption covered with a thin liquid layer, thereby simulating the epithelial layer of tissue. We compared the data with the theoretical predictions after collecting and analyzing, by means of our predictive model, ten sets of data from five patients and two controls. Initial results indicate that the model is effective in assessing the level of inflammation in patients.

The second generation of the device using flexible imaging fiber bundles has been designed and is under construction. In the new design, an imaging bundle replaces a set of optical fibers. Instead of yielding an output reading from each fiber sequentially, the device will use a CCD camera to record light distribution instantaneously. The new design offers two advantages over the existing device: shortening of the acquisition time and higher spatial resolution.

### Monitoring Blood Circulation in Kaposi's Sarcoma and Complex Regional Pain Syndrome Type I

*Hattery, Hekmat, Gabel, Togioka, Gandjbakhche; in collaboration with Yarchoan, Dionne*

We are investigating the use of three imaging modalities to quantify different parameters associated with blood circulation. The modalities are thermography, which provides a two-dimensional image of superficial skin temperatures and is based on the concept that higher temperatures occur in the skin superficial to veins that are involved in active transport of blood; laser Doppler imaging (LDI), which produces two-dimensional images of blood flow over a defined area at 690nm and 780nm; and hyperspectral imaging, which, designed in our unit, produces two-dimensional images at six wave lengths (700–1000nm) by using differential absorption algorithm (also devised by our unit) parameters related to the oxy-deoxy-hemoglobin ratio (i.e., oxygenation) and permits derivation of total blood content. Two studies are under way; the first deals with the skin disease Kaposi's sarcoma (KS) and the second with reflex sympathetic disorder (RSD). An NCI-sponsored clinical trial is evaluating the effectiveness of anti-angiogenetic drug treatment for KS. Because KS is a highly vascular tumor, angiogenesis and capillary permeability can play an important role in the development and progression of the disease. No noninvasive standard technique is available to assess the effect of anti-angiogenetic therapy on blood flow in KS. The purpose of the clinical trial is to investigate the applicability of the three noninvasive methods for the assessment of vascularity and vascular changes associated with KS. Eighteen patients have been investigated so far. A comparative image analysis of thermography, between the lesion and contralateral, lesion-free sites, shows that the temperature of the lesion sites is different from that of the contralateral side. Similar relationships are also observed in laser Doppler images. Preliminary results indicate that the techniques are useful for assessing vascularity and vascular changes associated with KS. Completion of the ongoing study should provide additional data to support the initial results.

RSD, currently known as Complex Regional Pain Syndrome (CRPS), is a chronic pain disorder involving the sympathetic nervous system. An NIDCR/NINDS clinical study is evaluating the response of patients experiencing CRPS type I to the drug Neurotropin. In this study, the nature of chronic neuropathic pain is assessed by asymmetric thermal pattern between pain and contralateral pain-free sites; comparisons between the site of pain and the corresponding contralateral side near surface blood flow; and sympathetic response of blood flow measured by LDI as well as by temperature patterns in pain and pain-free sites following cold stimulation. As an ongoing clinical trial, results have not been unblinded, and the effect of the drug compared with the placebo may not be assessed with the preliminary results.

## SELECTED PUBLICATIONS

1. Chernomordik V, Hattery D, Gannot I, Zaccanti G, Gandjbakhche AH. Analytical calculation of the mean time spent by photons inside an absorptive inclusion embedded in a highly scattering medium. J Biomed Opt. 2002;7:486-492.
2. Chernomordik V, Hattery D, Grosenick D, Wabnitz H, Rinneberg H, Thomas Moesta K, Schlag PM, Gandjbakhche AH. Quantification of optical properties of a breast tumor using random walk theory. J Biomed Opt. 2002;7:80-87.
3. Eidsath A, Chernomordik V, Gandjbakhche AH, Smith P, Russo A. Three-dimensional localization of fluorescent masses deeply embedded in tissue. Phys Med Biol. 2002;47:4079-4092.
4. Gannot I, Gannot G, Garashi A, Gandjbakhche AH, Buchner A, Keisari Y. Laser activated fluorescence measurements and morphological features–an in vivo study of clearance time of FITC tagged cell markers. J Biomed Optics. 2002;7:14-19.
5. Hassan M, Hattery D, Chernomordik V, Aleman K, Wyvill K, Merced L, Little R, Yarchoan R, Gandjbakhche AH. Non-invasive multi-modality technique to study angiogenesis associated with Kaposi's sarcoma. Proc IEEE EMBS BMES. 2002;in press.
6. Hattery D, Chernomordik V, Smith P, Pursley R, Atkinson J, Loew M, Mulshine J, Gandjbakhche A. Measuring oral inflammation in vivo with diffuse reflectance spectroscopy. Proc IEEE EMBS. 2002;in press.

7. Hattery D, Gerdelman K, Hekmat F, Chernomordik V, Smith P, Eidsath A, Atkinson J, Mulshine J, Gandjbakhche A. Using diffuse reflectance spectroscopy to quantify inflammation of the oral epithelium in vivo. In: Alfano R, ed. Proc SPIE Photonics West Conference: Progr Biomed Optics Imaging: Opt Biopsy. 2002:3:59-70.
8. Hattery D, Hassan M, Demos S, Gandjbakhche A. Hyperspectral imaging of Kaposi's sarcoma for disease assessment and treatment monitoring. Proc Appl Imagery Pattern Recognit. 2002;in press.

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# TISSUE BIOPHYSICS AND BIOMIMETICS

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Our goal is to understand structure-function relationships in tissues from a physical perspective. There is a unifying interest in understanding the role of water in different physiological processes, in particular, water-mediated interactions with biomolecules that allow cartilage to bear load or that play a role in nerve excitability. We also use nuclear magnetic resonance (NMR) measurements of water mobility in tissues to probe tissue microstructure and architectural organization.

## Diffusion Tensor Magnetic Resonance Imaging of the Brain

*Pierpaoli, Horkay; in collaboration with Pajevic, Barnett, Aldroubi, Shrager, Miranda, Cohen, Jones*

We are continuing to develop Diffusion Tensor Magnetic Resonance Imaging (DT-MRI) as a means to probe tissue microstructure and to assess and diagnose neurological and developmental disorders. DT-MRI measures a diffusion tensor of water on a pixel-by-pixel basis within tissue, both noninvasively and *in vivo*. It relates an effective diffusion tensor to the measured MR spin echo signal, estimating an effective diffusion tensor, $\boldsymbol{D}$, in each pixel from a set of diffusion-weighted MR images and then calculating and displaying information derived from $\boldsymbol{D}$, including local fiber-tract orientation, the mean-squared distance that water molecules diffuse in any given direction, the orientationally averaged mean diffusivity, and other intrinsic scalar invariant quantities that are independent of the laboratory coordinate system. These scalar parameters behave like quantitative histological or physiological "stains," yet they are "developed" without requiring exogenous contrast agents or dyes.

One example, the orientationally averaged diffusivity (or Trace[D]), has been the most successful imaging parameter proposed to date to identify ischemic tissue regions in the brain during and following an acute stroke. Moreover, we have shown that DT-MRI is effective in identifying white matter degeneration (Wallerian degeneration) associated with chronic stroke. Studies with kittens have also shown that DT-MRI is useful for following early developmental changes occurring in cortical gray and white matter. Such changes cannot be detected with other imaging methods. Developed by Sinisa Pajevic and Carlo Pierpaoli, a method to encode nerve fiber orientation in the brain by using color has allowed us to identify the main association, projection, and commissural white matter pathways in the human brain and even differentiate anatomical white matter pathways that have similar structure and composition but different spatial orientations. It has also allowed us to perform detailed studies of the brain's structural anatomy; previously, such studies could be performed only by using laborious, invasive histological methods. To assess anatomical connectivity between different functional regions in the brain, we have recently proposed and demonstrated a way to use DT-MRI data to trace out nerve fiber tract trajectories, which we call "fiber tractography." In this application, we compute the trajectory of a nerve fiber tract by continuously following the direction along which the apparent diffusivity is a maximum.

The development of DT-MRI also requires new mathematical, statistical, and image-processing concepts and constructs for analyzing the multidimensional data produced by the recently developed imaging method. Akram Aldroubi and Sinisa Pajevic have established a general mathematical framework for obtaining a continuous, smooth approximation to the discrete, noisy, measured diffusion tensor field data. The framework allows us to reduce the noise in our data and enables us to follow fibers more reliably. We have also derived the form of the parametric distribution governing the statistical variability of diffusion tensor data and have developed nonparametric (bootstrap) methods for determining features of the data's statistical distribution from experimental DT-MRI data. These developments are allowing us to apply powerful statistical hypothesis tests to address a wide variety of important biological and clinical questions that previously could be tackled only by using *ad hoc* methods. We are also developing novel image processing methods to enable us to perform quantitative longitudinal or multi-center DT-MRI studies. Gustavo Rohde has been developing methods to warp and register multi-dimensional DT-MRI data sets. To ensure that the DT-MRI acquisitions obtained with different scanners and at different sites are quantitative and of high quality, Ferenc Horkay is developing new polymeric phantoms with which we can calibrate DT-MRI measurement systems. Collectively, these developments are enhancing the utility and broadening the scope of applications of DT-MRI in medicine and biology. Finally, fundamental connections have been established between DT-MRI and a widely used methodology for studying material microstructure and morphology called q-space MRI. We will continue to explore these connections in the coming years.

### Physical-Chemical Aspects of Cell and Tissue Excitability

*Horkay, Basser; in collaboration with Tasaki*

Excitability of cells and tissues is an essential physiological function that allows organisms to sense their environment and respond to it. The primary goal of our work is to explain key physical-chemical features of cell and tissue excitability, many aspects of which are still poorly understood. Widely accepted theories of nerve excitability fail to explain several anomalous phenomena that we have both observed and shown to be necessary for excitation to occur. These phenomena include volume and temperature changes of the superficial protoplasmic layer of nerve axons, which coincide with the action potential waveform. These changes also coincide with a phase transition that occurs in nerve cells, fibers, and synapses and that is caused by the exchange of divalent cations, such as calcium, with monovalent cations, such as sodium and potassium. Our previous experiments with perfused axons clearly implicate divalent/monovalent cation exchange as a mechanism by which nerve fibers can be excited in an "all or none" manner. To understand the physical-chemical basis of these temperature and volumetric changes, particularly how divalent/monovalent cation exchange can induce such changes in biomolecular assemblies, we are studying these processes in synthetic "biomimetic" anionic polymer gels under quasi-physiological solution conditions. An advantage of studying the behavior of these gel model systems is that, unlike in the case of living tissue, their structure, composition, and interactions among their components can be carefully controlled. In particular, in synthetic polyacrylate gels, Ferenc Horkay has observed that minute changes in the concentration of divalent cations in the surrounding liquid can induce significant changes in chain stiffness in the gel, even if ion binding is weak and completely reversible. Various physical-chemical and polymer physics–based techniques, including neutron scattering, osmotic swelling, and mechanical loading, provide complementary information with which to study these biologically relevant phenomena over a wide range of length scales. These basic studies are leading to a deeper understanding of the physical mechanisms underlying nerve excitation.

### Functional Properties of Extracellular Matrix

*Horkay, Basser; in collaboration with Amis*

The collagen network plays a critical role in determining functional properties of cartilage and other extracellular matrices. The collagen network exerts a retractive stress on the osmotically active proteoglycans that are trapped within it in much the same way that a balloon's elastic membrane exerts hydrostatic pressure on the gas contained within it. Until recently, however, it was not possible to measure the retractive stress of the collagen network independently of other constituents within the extracellular matrix. Previously, we devised a new methodology to determine this structural property of the collagen network, a methodology that involves (1) modeling the cartilage tissue matrix as a composite material consisting of two distinct phases: a collagen network and a proteoglycan (PG) solution trapped within it; (2) applying various known levels of equilibrium osmotic stress; and (3) using physical-chemical principles and independent experiments to determine useful "pressure-volume" relations for both the PG and collagen phases independently. In pilot studies, we used this approach to determine pressure-volume curves for the collagen network and the PG phases in native and in trypsin-treated normal human cartilage specimen as well as in cartilage specimens from osteoarthritic (OA) joints. In both normal and trypsin-treated specimens, collagen network stiffness appeared unchanged, whereas collagen network stiffness decreased in the OA specimen. Our findings highlight the critical role of the collagen network in limiting normal cartilage hydration and in ensuring a high PG concentration in the matrix, both of which are essential for effective load bearing in cartilage but are lost in OA. The data also suggest that the loss of collagen network stiffness, and not the loss or modification of PGs, may be the incipient event leading to the subsequent disintegration of cartilage observed in OA.

More recently, Ferenc Horkay has developed a prototype instrument to study osmotic properties of extremely thin tissue sections. The instrument will permit us to obtain a profile of the functional properties of cartilage as a function of depth from the joint's articular surface. Horkay is also developing novel spectroscopic methods (both light scattering and neutron scattering) to investigate interactions between water, ions, and extracellular matrix constituents in various tissue specimens.

## SELECTED PUBLICATIONS

1. Alexander DC, Pierpaoli C, Basser PJ, Gee JC. Spatial transformations of diffusion tensor magnetic resonance images. IEEE Trans Med Imaging. 2001;20:1131-1139.
2. Basser PJ, Diffusion MRI. In: Atlas S, ed. Brain and spine. Third edition. New York: Lippincott, Williams and Wilkins, 2001;197-214.
3. Basser PJ. Relationships between diffusion tensor and q-space MRI. Magn Reson Med.2002;47:392-397.
4. Basser PJ, Jones DK. Diffusion-tensor MRI: theory, experimental design, and data analysis. NMR Biomed. 2002;15:1-13.
5. Basser PJ, Pajevic S, Pierpaoli C, Aldroubi A. Fiber tract following in the human brain using DT-MRI data. IEICE T Inf Syst. 2002;E85D:15-21.
6. Dimitriadis EK, Horkay F, Maresca J, Kachar B, Chadwick RS. Determination of elastic moduli of thin layers of soft material using the atomic force microscope. Biophys J. 2002;82:2798-2810.
7. Hecht A, Horkay F, Geissler E. Neutron scattering investigations on a bimodal polymer gel. J Phys Chem B. 2001;105:5637-5642.
8. Horkay F, Basser PJ, Hecht A, Geissler E. Ion-exchange induced change in the structure and osmotic properties of sodium polyacrylate hydrogels. Macromol Sym. 2001;171:201-208.
9. Horkay F, Basser PJ, Hecht AM, Geissler E. Calcium-induced volume transition in polyacrylate hydrogels swollen in physiological salt solutions. Macromol Biosci. 2002;2:207-213.

10. Horkay F, Hecht A. Structure of polymer solutions and gels containing fillers. Macromol Sym. 2001;171:171-180.
11. Horkay F, Hecht A, Basser PJ, Geissler E. Comparison between neutral gels and neutralized polyelectrolyte gels in the presence of divalent cations. Macromolecules. 2001;34:4285-4287.
12. Horkay F, Tasaki I, Basser PJ. Effect of monovalent-divalent cation exchange on the swelling of polyacrylate hydrogels in physiological salt solutions. Biomacromolecules. 2001;2:195-199.
13. Pajevic S, Aldroubi A, Basser PJ. A continuous tensor field approximation of discrete DT-MRI data for extracting microstructural and architectural features of tissue. J Magn Reson. 2002;154:85-100.
14. Pierpaoli C, Barnett A, Pajevic S, Chen R, Penix LR, Virta A, Basser PJ. Water diffusion changes in Wallerian degeneration and their dependence on white matter architecture. Neuroimage. 2001;13:1174-1185.
15. Potter K, Leapman RD, Basser PJ, Landis WJ. Cartilage calcification studied by proton nuclear magnetic resonance microscopy. J Bone Miner Res. 2002;17:652-660.

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# GENES THAT FASHION THE MOUSE EMBRYO

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We study the molecular genetics of patterning, axis formation, and organogenesis in the developing mouse embryo. Our current work examines key control elements of these processes and shows the emergence of a scenario of protein-protein interactions that controls every aspect of the body plan. We have used a loss-of-function approach to identify basic components of protein complexes that activate target genes during the course of development.

## Transcriptional Cofactors

*Chen, Grinberg, Huang, Mukhopadhyay, Schindler, Teufel, Westphal; in collaboration with Agulnick, Bayarsaihan, Dawid, Dorward, Downs, Hukriede, Kennison, Ogryzko, Podtelejnikov, Segal, Yamashita*

Over the years, our laboratory has carried out extensive studies on LIM-homeodomain proteins. These *Lhx* gene products are important transcriptional regulators of invertebrate and vertebrate embryonic development. They are involved in early patterning events, the development of the nervous system, and organogenesis. Their action is facilitated by co-factors that were identified by their ability to dimerize and to bind to the LIM domain, a specialized zinc-finger structure present in *Lhx* gene products and in a number of other nuclear proteins. In vertebrates, these LIM-binding co-factors are known as Ldb, Nli, or Clim; in *Drosophila* as Chip; and in *C. elegans* as Ldb1. Protein-protein interactions involving Ldb/Nli/Clim (henceforth referred to as Ldb) and Chip are not restricted to LIM domain–containing factors but can involve a host of other transcriptional regulators as well. Ample evidence supports the notion that the Ldb and Chip co-factors are essential components of developmental programs controlled by transcriptional regulators. More recently, the Rlim cofactor was identified and shown to control transcription factors negatively by targeting Ldb proteins for degradation. Furthermore, competition of transcription factors for binding to Chip or Ldb can alter developmental cell fates.

The mammalian Ldb1 protein is found in multi-protein complexes containing various combinations of LIM-homeodomain, LIM-only, bHLH, GATA, and Otx transcription factors. These proteins exert key functions during embryogenesis. We have established that targeted deletion of the *Ldb1* gene in mice results in a pleiotropic phenotype, revealing fundamental roles of *Ldb1* in important developmental decisions. First, there is no heart anlage in the *Ldb1*-null mutant embryo. Our data suggest that *Ldb1* gene function is essential for proper allocation of cardiac mesoderm to the heart field in the gastrulating embryo. Abnormal migration of the heart mesoderm in the mutants may abolish inductive interactions of the tissue with anterior endoderm and thus prevent heart development. In addition, head development is severely curtailed in the mutant, and head structures are truncated anterior to the hindbrain. In about 40 percent of the mutants, posterior axis duplication is observed. The expression of several Wnt inhibitors is curtailed in the mutant, suggesting that Wnt pathways may be involved in axial patterning events regulated by *Ldb1*. Abnormal organizer gene expression during gastrulation may account for the observed axis defects in the *Ldb1* mutant embryos. Finally, we observed in the *Ldb1*-null mutant conceptus severe defects in mesoderm-derived extraembryonic structures, including the allantois, blood islands of the yolk sack, primordial germ cells, and the amnion, attesting to a pivotal role of Ldb1 in mesoderm formation.

To identify additional components of Ldb/Chip-containing nuclear protein complexes, we generated HeLa cells that express FLAG and HA epitope–tagged mouse Ldb1, purified nuclear complexes with the aid of the tags, and identified constituent proteins by mass spectrometry of tryptic peptides. We identified Ssdp proteins (previously described as sequence-specific, single-stranded-DNA-binding proteins) as components of Ldb1-associated nuclear complexes in these cells. Ssdp proteins are associated with Ldb1 in a variety of additional mammalian cell types. The association is specific, does not depend on the presence of nucleic acids, and is functionally significant. Genes encoding Ssdp proteins are well conserved in evolution from *Drosophila* to humans. Whereas the vertebrate *Ssdp* gene family has several closely related members, the *Drosophila* Ssdp gene is unique. In *Xenopus*, Ssdp encoded by *Drosophila Ssdp* or mouse *Ssdp1* mRNA enhances axis induction by *Ldb1* in conjunction with the LIM-homeobox gene *Xlim1*. Furthermore, we were able to demonstrate an interaction between *Ssdp* and *Chip* (the fly homolog of *Ldb1*) in *Drosophila* wing development. These findings indicate functional conservation of Ssdp as a co-factor of Ldb1 in transcriptional regulation during invertebrate and vertebrate development.

### Novel Genes

*Hasuike, Malik, Miyamoto, Mukhopadhyay, Teufel, Westphal, Zhao; in collaboration with Dawid, Fiorenza, Kawahara*

Our laboratory has identified a number of novel genes involved in the development of the mouse embryo. We detected the first two, *Thg-1pit* and *MPPα2*, in a screen for genes that are differentially expressed in the developing pituitary of wild-type and *Lhx3*-null mutant embryos. The genes are active at early stages of pituitary development. A third gene, *Mbx*, encodes a homeodomain transcription factor that is initially restricted to the midbrain region of the mouse embryo but later extends to the developing forebrain. Finally, we cloned *Frcp1* and *Frcp2*, two genes that are also primarily expressed in the brain. These genes contain an FNIII motif characteristic for a variety of receptor and cell adhesion proteins.

## SELECTED PUBLICATIONS

1. Chen L, Segal D, Hukriede N, Podtelejnikov A, Bayarsaihan D, Kennison JA, Ogryzko V, Dawid IB, Westphal H. Ssdp proteins interact with the LIM-domain binding protein Ldb1 to regulate development. Proc Natl Acad Sci USA. 2002;99:14320-14325.
2. Fiorenza MT, Mukhopadhyay M, Westphal H. Expression screening for Lhx3 downstream genes identifies Thg-1pit as a novel gene involved in pituitary development. Gene. 2001;278:125-130.
3. Miyamoto T, Fiorenza MT, Zhao Y, Hasuike S, Westphal H. Molecular cloning and expression analysis of MPPα-2, a novel mouse transcript detected in a differential screen of pituitary libraries. Biochim Biophys Acta. 2002;1577:109-112.
4. Miyamoto T, Kawahara A, Teufel A, Mukhopadhyay M, Zhao Y, Dawid IB, Westphal H. Mbx, a novel mouse homeobox gene. Dev Genes Evol. 2002;212:104-106.
5. Mukhopadhyay M, Shtrom S, Rodriguez-Esteban C, Chen L, Tsukui T, Gomer L, Dorward DW, Glinka A, Grinberg A, Huang SP, Niehrs C, Belmonte JC, Westphal H. Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. Dev Cell. 2001;1:423-434.

6. Paylor R, Zhao Y, Libbey M, Westphal H, Crawley JN. Learning impairments and motor dysfunctions in adult Lhx5-deficient mice displaying hippocampal disorganization. Physiol Behav. 2001;73:781-792.
7. Teufel A, Malik N, Mukhopadhyay M, Westphal H. Frcp1 and Frcp2, two novel fibronectin type III repeat containing genes. Gene. 2002;297:79.
8. Westphal H. Genes that fashion the pituitary gland. Mol Cell Endocrinol. 2002;197:45-46.
9. Zhao Y, Westphal H. Homeobox genes and human genetic disorders. Curr Mol Med. 2002;2:13-23.

## COLLABORATORS

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# SIGNALS REGULATING T CELL DEVELOPMENT

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Our research is directed at understanding the cellular and genetic events that regulate T lymphocyte development. Current studies focus on the role of T lymphocyte signal-transducing molecules in immature T lymphocyte (thymocyte) selection, a process essential for formation of the mature T cell repertoire. To analyze the function of specific signal-transducing proteins in T lymphocyte development, we use transgenic and gene-targeting methods to create over-expression, dominant-negative, and loss-of-function mutants in mice. In addition, we are employing molecular genetic techniques to identify and characterize the function of novel genes that are expressed in T lymphocytes.

### Role of T Cell Antigen Receptor Signalling in Thymocyte Development

*Frolova, El-Khoury, Love; in collaboration with Shores*

A major theme of our research has been the role of T cell antigen receptor (TCR) signal transduction in thymocyte development. Signal transduction sequences (termed immunoreceptor tyrosine-based activation motifs; ITAMs) are contained within four distinct subunits of the multimeric TCR complex (zeta, CD3-gamma, CD3-delta, CD3-epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement and function to recruit signalling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell activation cascade. Though conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be regulated, in part, by distinct ITAMs. To determine if TCR signal–transducing subunits perform distinct or analogous functions in development, we generated zeta-deficient and CD3-epsilon–deficient mice by gene targeting. We genetically reconstituted these mice with transgenes encoding wild-type or signalling-deficient (ITAM-mutant) forms of zeta and CD3-epsilon and characterized the developmental and functional consequences of these alterations for TCR signalling. The results of the studies demonstrate that TCR-ITAMs are functionally equivalent but act in concert to amplify TCR signals. TCR signal amplification proved to be critical for thymocyte selection, the process by which potentially useful immature T cells are instructed to survive and differentiate further (positive selection) and by which potentially auto-reactive cells that may cause auto-immune disease are deleted in the thymus (negative selection). Thus, the multi-subunit structure of the TCR may have evolved to enable complex organisms to develop a self-restricted T cell repertoire.

### Mechanism of CD5-Mediated TCR Signal Inhibition

*Park, Love*

The cell surface protein CD5 has been shown to regulate TCR signalling negatively and to participate in thymocyte selection. Examination of CD5 expression during T cell development revealed that surface levels of CD5 are regulated by TCR signal intensity and by the affinity of the TCR for ligands in the thymus that mediate selection. To determine if the ability to regulate CD5 expression is important for thymocyte selection, we generated transgenic mice that constitutively express high levels of CD5 throughout development. Over-expression of CD5 significantly impaired positive selection of some thymocytes (those that would normally express low levels of CD5) but not others (those that would normally express high levels of CD5). These findings support a role for CD5 in modulating TCR signal transduction and thereby influencing the outcome of thymocyte selection. The ability of individual thymocytes to regulate CD5 expression represents a mechanism for "fine tuning" the TCR signalling response during development. Our results indicate that the potential for signal modulation may be particularly useful for generating the maximum possible TCR diversity in the mature T cell repertoire. Given that a probable mechanism for CD5 function involves the activation-induced binding of regulatory molecule(s) to sequences within the CD5 cytoplasmic domain, we generated transgenic mice that express a tail-less form of CD5 (mCD5). We then used both the intact and mCD5 transgenes to reconstitute CD5 surface expression in CD5$^{-/-}$ mice. The experiments revealed a critical function for the cytoplasmic domain in CD5 signalling. The laboratory is currently attempting to identify molecules that interact with CD5 in an effort to determine how CD5 regulates signal transduction by the TCR.

### Role of LAT in T Cell Development

*Park, Love; in collaboration with Samelson, Sommers, Shores*

Linker for activation of T cells (LAT) is an integral membrane protein that functions as a critical adaptor, linking the T cell antigen receptor (TCR) to multiple down-stream signalling pathways required for T cell activation. The distal four tyrosines in LAT (tyr$^{136}$, tyr$^{175}$, tyr$^{195}$, tyr$^{235}$) are necessary and sufficient for LAT activity in T cells, which includes activation of the calcium and MAP Kinase (MAPK) down-stream signalling pathways. These signalling pathways are also activated by a large number of other receptors and are required for the development and function of many different cell types. Thus, their inactivation in all cells would likely result in embryonic lethality. However, by mutating specific LAT tyrosines, we have been able to uncouple the TCR from down-stream signalling pathways in T cells without affecting the ability of other receptors or cells to use these pathways. We generated "knock-in" mutant mice that express LAT proteins containing single or multiple tyrosine-phenylalanine mutations of the four critical tyrosine residues. Knock-in mice that express the wild-type version of the protein exhibited normal T cell development, thereby validating the targeting strategy. Inactivation of all four distal LAT tyrosines yielded a null phenotype, demonstrating the critical role of these residues for T cell development. Surprisingly, knock-in mutation of the first tyr residue (tyr$^{136}$) resulted in a profound fatal lymphoproliferative disorder characterized by expansion and multi-tissue infiltration of CD4$^{+}$ T cells. Consistent with previous data demonstrating that tyr$^{136}$ preferentially binds to phospholipase C-gamma, examination of the signalling response of T cells from these mice revealed a severe defect in TCR-induced/phospholipase C-gamma–mediated calcium flux. However, MAP kinase signalling was intact in these cells, indicating that the TCR was specifically uncoupled from the calcium but not from the MAPK pathway. The results reveal a critical role for LAT in coordinating down-stream signals initiated by TCR engagement and demonstrate that this function is essential for normal T cell homeostasis.

### Structure and Signalling Potential of the Gamma/Delta TCR Complex

*Hayes, Love; in collaboration with Fowlkes, Laky*

Most vertebrate species contain two separate lineages of T cells that are distinguished by the clonotype-specific chains contained within their

TCRs: alpha/beta-T cells and gamma/delta-T cells. Although the alpha/beta-TCR has been extensively characterized, much less is known about the structure or function of the gamma/delta-TCR. We found that the subunit composition of the gamma/delta-TCR differs fundamentally from that of the alpha/beta-TCR in that it lacks a major subunit of the alpha/beta-TCR, the CD3delta chain. Interestingly, signal transduction by the gamma/delta-TCR was consistently superior to that by the alpha/beta-TCR as assessed by several criteria. These results demonstrate a major difference in the subunit structure of the alpha/beta- and gamma/delta-TCRs. Moreover, our data suggest that the structural difference may influence the signalling potential of the TCR complex and have important functional consequences on T cell activation.

### Role of the Chemokine Receptor CCR9 in T Cell Development

*Uehara, Love; in collaboration with Farber*

T cell development continues into adulthood and requires the periodic migration of T-progenitor cells from the bone marrow to the thymus. The ordered progression of thymocytes through distinct stages of development is also associated with migration into and between different thymic microenvironments where they are exposed to different growth factors and signals. Chemokines are a group of small, structurally related molecules that regulate trafficking of leukocytes through interactions with a subset of seven-transmembrane, G protein–coupled receptors. The chemokine CCL25 is highly expressed in the thymus and small intestine, the two known sites of T lymphopoesis. The receptor for CCL25, CCR9, is expressed on the majority of thymocytes, raising the possibility that CCR9 and its ligand play an important role in thymocyte development. To investigate the role of CCR9 during lymphocyte development, we generated CCR9-deficient (CCR9$^{-/-}$) and CCR9-transgenic mice. Surprisingly, both T cell and B cell development appeared normal in CCR9$^{-/-}$ mice. However, competitive bone marrow transplantation experiments demonstrated that CCR9$^{-/-}$ bone marrow cells had a markedly reduced capacity to repopulate the thymus compared with bone marrow cells from CCR9$^{+/+}$ mice. Overexpression of CCR9 in transgenic mice inhibited early thymocyte development and blocked the normal migration of immature thymocytes within the thymus. These results demonstrate that CCR9 participates in regulating both the migration of progenitor cells to the thymus and the migration of developing thymocytes within the thymus.

## SELECTED PUBLICATIONS

1. Azzam HS, DeJarnette JB, Huang K, Emmons R, Park CS, Sommers CL, El-Khoury D, Shores EW, Love PE. Fine tuning of TCR signaling by CD5. J Immunol. 2001;166:5464-5472.

2. Feng C, Woodside KJ, Vance BA, El-Khoury D, Canelles M, Lee J, Gress R, Fowlkes BJ, Shores EW, Love PE. A potential role for CD69 in thymocyte emigration. Int Immunol. 2002;14:535-544.

3. Hayes SM, Love PE. Distinct structure and signaling potential of the gamma delta TCR complex. Immunity. 2002;16:827-838.

4. Sommers CL, Menon RK, Grinberg A, Zhang W, Samelson LE, Love PE. Knock-in mutation of the distal four tyrosines of linker for activation of T cells blocks murine T cell development. J Exp Med. 2001;194:135-142.

5. Sommers CL, Park CS, Lee J, Feng C, Fuller CL, Grinberg A, Hildebrand JA, Lacana E, Menon RK, Shores EW, Samelson LE, Love PE. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science. 2002;296:2040-2043.

6. Uehara S, Grinberg A, Farber JM, Love PE. A role for CCR9 in T lymphocyte development and migration. J Immunol. 2002;168:2811-2819.

7. Uehara S, Song K, Farber JM, Love PE. Characterization of CCR9 expression and CCL25/thymus-expressed chemokine responsiveness during T cell development: CD3(high)CD69+ thymocytes and gammadeltaTCR+ thymocytes preferentially respond to CCL25. J Immunol. 2002;168:134-142.

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# MOLECULAR GENETICS OF AN IMPRINTED GENE CLUSTER ON MOUSE DISTAL CHROMOSOME 7

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Genomic imprinting is an unusual form of gene regulation in which expression of an allele is restricted in accordance with its parental origin. Thus, some genes, such as *H19*, are expressed only from the maternally inherited chromosome while the paternal allele is silent. In contrast, the neighboring *Igf2* gene is expressed almost exclusively from the paternal allele. Imprinted genes are not randomly scattered throughout the chromosome but rather are localized in discrete clusters. One cluster of imprinted genes is on the distal end of mouse chromosome 7. The syntenic region in humans (11p15.5) is highly conserved in gene organization and expression patterns. Mutations disrupting the normal patterns of imprinting at the human locus are associated with a developmental disorder, Beckwith Wiedemann syndrome, and with many types of tumors. In addition, inherited cardiac arrhythmia is associated with mutations in the maternal-specific *Kcnq1* gene. Our studies involve the use of imprinting as a tool for understanding fundamental features of epigenetic regulation of gene expression and rely on the mouse system for generating animal models for the several inherited disorders associated with this region, specifically the effect of β-adrenergic–mediated stress on the cardiac phenotype.

### Molecular Basis for Allele-Specific Expression of the Mouse *H19* and *Igf2* Genes

*Park, Boe, Pfeifer*

Our studies of imprinting focus on expression of the *H19* and *Igf2* genes. Paternally expressed *Igf2* lies about 70 kb upstream of the maternal-specific *H19* gene. By using cell culture systems and conducting transgene and knock-out experiments *in vivo*, we have identified the enhancer elements responsible for activation of the two genes. The elements are largely shared and located downstream of the *H19* gene. Parent-of-origin–specific expression of both genes depends on a shared element (called the *H19DMR*) located just upstream of the *H19* promoter and thus juxtaposed between the *Igf2* gene and the shared enhancers. The CpG sequences within this element are methylated specifically on the paternally inherited chromosome. Our conditional ablation of this element *in vivo* demonstrates that the non-methylated *H19DMR* (i.e., the copy on the maternal chromosome) is continually required for silencing of the maternal *Igf2* allele. Knock-in experiments demonstrate that the *H19DMR* contains a transcriptional insulator that is inactivated, and thus permissive for enhancer-promoter interactions, by DNA methylation. Therefore, on the non-methylated maternal chromosome, the active insulator within the *H19DMR* prevents activation of *Igf2* by the downsteam enhancers. Methylation of the paternal chromosome inactivates the insulator and permits *Igf2* expression. Unexplained by this model is the effect of several small *DMRs* proximal to the *Igf2* transcription unit. Current studies are investigating the mechanistic significance of these elements. Imprinting of *H19* occurs via a distinct genetic mechanism. The conditional ablation of the *H19DMR* indicates that it is not continuously required for silencing the paternal allele. Rather, the *H19DMR* is required early in development to establish an epigenetic state at the *H19* promoter that itself prevents transcription. Current studies provide evidence that the epigenetic program includes, but is not solely, the hypermethylation of the *H19* promoter.

To determine precisely the elements that are necessary and sufficient for imprinting at the locus, we have moved the *H19DMR* and mutated derivatives to a normally unimprinted locus. Mice carrying the mutation are currently under analysis, and results indicate that both forms of gene regulation, the developmentally dependent modification of neighboring sequences and transcriptional insulation, can be examined by the above approach.

### Mouse Models for Inherited Long QT Syndrome

*Casimiro, Pfeifer; in collaboration with Ebert, Knollman*

Inherited long QT syndrome (LQTS) is characterized by an abnormal electrocardiogram indicative of repolarization defects and can result in syncope or sudden death. Romano-Ward syndrome (RWS) patients inherit the LQTS disorder generally as a dominant phenotype and show no other traits. Jervell and Lange-Nielsen syndrome (JLNS) patients display profound congenital deafness in addition to the LQTS, and both phenotypes are recessive. We have generated several mutations in the mouse *Kcnq1* gene to model the human diseases. Ablation of the gene results in vestibular and auditory defects. Histological analyses suggest that the defects are attributable to a deficiency in the $K^+$ recycling pathway that is crucial for generating endolymph, the specialized fluid bathing the inner hair cells. When measured by ECG, *in vivo* tracings of mutant mice are indicative of profound defects in cardiac repolarization. However, the defects are not noted in isolated hearts *ex vivo*, demonstrating that the Kcnq1 protein plays a central role in mediating critical extracardiac signals. Further analyses demonstrate that Kcnq1 function is specifically required to modulate cardiac function in the presence of β-adrenergic stimulation.

We have also generated three point mutations to model RWS. We have analyzed mutations in the central pore region and in the sixth membrane-spanning domain. The phenotypes of these mutations are each a distinct subset of those seen in the null mutation and thus demonstrate that the Kcnq1 protein plays distinct roles in the heart versus the inner ear and in various aspects of cardiac function. While inherited LQTS is relatively rare, our genetic models represent excellent paradigms for addressing mechanisms of acquired LQTS, the single largest cause of death in Western societies.

### Beta-Adrenergic Hormone Synthesizing Cells and Development of the Cardiac Conduction System

*Boe, Pfeifer*

During early development, the heart is the primary (and probably the only) site of synthesis of the β-adrenergic hormones, norepinephrine and epinephrine. This cardiac-specific synthesis is transient and disappears by mid-gestation. Intriguingly, the cells synthesizing the β-adrenergic hormones are located in positions that predict the location of the developing cardiac conduction network. To understand the fate of cells that synthesize these hormones, we generated a mouse with a mutated *Pnmt* locus such that that cre recombinase enzyme is synthesized in any cell normally making epinephrine. (*Pnmt* encodes phenylethanolamine N-methyltransferase, the enzyme that converts norepinephrine to epinephrine.) When crossed with appropriate tester strains, *Pnmt*-expressing cells and their descendants become β-galactosidase–positive and thus can be readily identified and isolated. Early analyses indicate that epinephrine is synthesized by cells that specifically give rise to cardiac conduction cells, albeit a subset of such cells. Ongoing experiments will characterize cell types. Parenthetically, our studies have generated a mouse that has no epinephrine but shows normal levels of norepinephrine, thus allowing dissection of the specific roles of these two hormones.

## SELECTED PUBLICATIONS

1. Casimiro M, Knollman B, Ebert S, Vary JC, Huang SP, Grinberg A, Pfeifer K. Targeted disruption of the *Kcnq1* gene produces a mouse model of Jervell and Lange-Nielsen syndrome. Proc Natl Acad Sci USA. 2001;98:2526-2531.
2. Kaffer CR, Grinberg A, Pfeifer K. Regulatory mechanisms at the mouse *Igf2/H19* locus. Mol Cell Biol. 2001;21:8189-8196.
3. Kaffer CR, Srivastava M, Park KY, Ives E, Hsieh S, Batlle J, Grinberg A, Huang SP, Pfeifer K. A transcriptional insulator at the *H19/Igf2* locus. Genes Dev. 2000;14:1908-1919.
4. Pfeifer K. Mechanisms of genomic imprinting. Am J Hum Genet. 2000;67:777-787.
5. Srivastava M, Frolova E, Rottinghaus B, Boe SP, Grinberg A, Lee E, Love PE, Pfeifer K. Imprint control element mediated secondary methylation imprints at the Igf2/H19 locus. J Biol Chem. 2002 Sept 20[epub ahead of print].
6. Srivastava M, Hsieh S, Grinberg A, Willimans-Simon L, Huang SP, Pfeifer K. *H19* and *Igf2* monoallelic expression is regulated in two distinct ways by a shared *cis* acting regulatory region upstream of *H19*. Genes Dev. 2000;14:1186-1195.

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# MOLECULAR GENETICS OF EMBRYOGENESIS IN *XENOPUS* AND ZEBRAFISH

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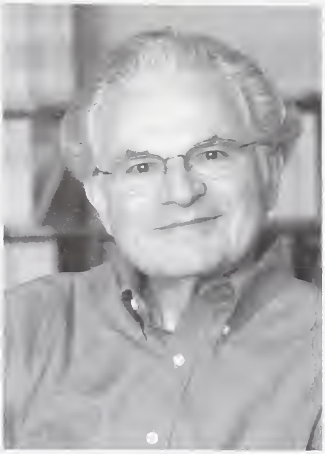

Using the frog *Xenopus laevis* and the zebrafish *Danio rerio* as experimental systems, the laboratory is engaged in studies of molecular-genetic mechanisms of early vertebrate development.

### The Small GTPases Rho and Rac Are Components of the Noncanonical Wnt Pathway in Controlling Gastrulation Movements

*Habas, Dawid; in collaboration with He*

The work of many laboratories has demonstrated a role for Wnt signalling in gastrulation movements. Wnt signals are transduced by three distinct pathways in different biological contexts. Gastrulation movements require the so-called noncanonical pathway that is mediated by frizzled receptors and the Dishevelled (Dsh) protein but does not result in activation of β-catenin. In studying the mechanism of signal transduction downstream of Dsh, we have shown previously that the small GTPase Rho is activated as a response to Wnt signalling and that the novel formin-family protein, Daam1, is required in this process (Habas *et al.*, 2001). Rho-family GTPases are known regulators of cell physiology with important functions in cytoskeletal dynamics and effects on gene activation. More recent studies have demonstrated that activation of Rho family GTPase Rac is also required for gastrulation movements. Rac activation is mediated by Wnt signalling through the function of Dsh, but the structural requirements in Dsh for Rho and Rac activation differ. Inhibition of either Rho or Rac activation alone leads to failure of gastrulation, indicating that the independent activation of both GTPases downstream of Wnt signalling is required for early development.

### The Role of *Lim1* in Gastrulation

*Hukriede, Habas, Dawid, in collaboration with Weeks, Tam*

The *Lim1* gene encodes a LIM-homeodomain protein that is expressed in the organizer region of all vertebrate embryos. A knock-out in the mouse, generated by Shawlot and Behringer, has shown that the *Lim1* gene is required for head formation. Further mechanistic studies carried out in *Xenopus* used antisense oligonucleotide depletion techniques developed by Weeks and Dagle, University of Iowa. *Lim1*-depleted frog embryos failed to form head structures, providing a phenocopy of *Lim1*-deficient mice. Most organizer-specific gene expression was retained in the affected embryos. but cell movements characteristic for gastrulation were impeded. The defect was traced to impaired expression of paraaxial protocadherin (PAPC), an adhesion molecule shown by De Robertis and colleagues, UCLA, to be involved in gastrulation. This defect in PAPC expression correlated with a failure of Rho activation on the dorsal side of the embryo. Rho activation has previously been shown to be required for gastrulation movements, as discussed above. We conclude that a loss of PAPC expression, directly and indirectly through Rho activation, affects gastrulation movements in *Lim1*-depleted embryos. Complementary studies in the mouse showed that *Lim1*$^{-/-}$ cells fail to participate in gastrulation movements when transplanted into wild-type embryos. Together, these studies indicate that control of gastrulation movements is a major function of the *Lim1* gene in different vertebrate embryos.

### Sef, a Novel Feedback Inhibitor of Fgf Signalling

*Tsang, Kudoh, Dawid; in collaboration with Friesel*

Fibroblast growth factors constitute a family of signalling factors that regulate many different developmental and physiological processes, and mutations in Fgf receptors are associated with several disorders in humans. The Fgf signal transduction pathway has been studied extensively in many systems, yet it was possible to isolate a novel feedback inhibitor of the pathway by observing a gene with Similar Expression to Fgfs (Sef) in zebrafish. The group of C. and B. Thisse, University of Strasbourg, independently identified Sef, which is a transmembrane protein whose expression is controlled by Fgf signalling. Its role, as studied by gain-of-function and loss-of-function approaches, is to attenuate the Fgf signal in the embryo. Thus, Sef joins the ranks of many factors identified in various pathways that have a role in limiting the extent or range of their cognate signalling pathway within the organism.

### Interplay of Different Signalling Pathways in Patterning the Neural Ectoderm in Zebrafish

*Kudoh, Dawid; in collaboration with Wilson*

In a model proposed some time ago by the great embryologist Pieter Nieuwkoop, the nervous system is first induced by action of the organizer with anterior characteristics and is subsequently patterned by influences from the mesoderm that give a portion of the developing neural ectoderm more posterior characteristics. Many studies have implicated three classes of signalling factors, Fgf, Wnt, and retinoic acid (RA), in posteriorization of the neural ectoderm. We have studied the regulatory interrelationships between these signalling factors in the zebrafish. By a combination of gain-of-function and loss-of-function experiments, we determined the effects of each of these signals on the neural ectoderm as well as the relationships between them in controlling neural patterning. From these data, we conclude that each of the three signalling pathways alone is capable of inhibiting anterior development in the neural ectoderm, but induction of posterior development has an obligatory requirement for RA action. Thus, RA function is epistatic to both Wnt and Fgf signalling in posteriorization of the neural ectoderm, but all three pathways must interact in precise ways for the generation of the normal anteroposterior pattern of the zebrafish nervous system.

## SELECTED PUBLICATIONS

1. Andreazzoli M, Broccoli V, Dawid IB. Cloning and expression of noz1, a zebrafish zinc finger gene related to Drosophila nocA. Mech Dev. 2001;104:117-120.
2. Chen L, Segal D, Hukriede NA, Podtelejnikov A, Bayarsaihan D, Kennison JA, Ogryzko V, Dawid IB, Westphal H. Ssdp proteins interact with the LIM-domain binding protein Ldb1 to regulate development. Proc Natl Acad Sci USA. 2002;99:14320-14325.
3. Dawid IB, Chitnis AB. Lim homeobox genes and the CNS: a close relationship. Neuron. 2001;30:301-303.
4. Habas R, Kato Y, He X. Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. Cell. 2001;107:843-854.
5. Hong SK, Kim CH, Yoo KW, Kim HS, Kudoh T, Dawid IB, Huh TL. Isolation and expression of a novel neuron-specific onecut homeobox gene in zebrafish. Mech Dev. 2002;112:199-202.

6. Hukriede N, Fisher D, Epstein J, Joly L, Tellis P, Zhou Y, Barbazuk B, Cox K, Fenton-Noriega L, Hersey C, Miles J, Sheng X, Song A, Waterman R, Johnson SL, Dawid IB, Chevrette M, Zon LI, McPherson J, Ekker M. The LN54 radiation hybrid map of zebrafish expressed sequences. Genome Res. 2001;11:2127-2132.
7. Kawahara A, Chien CB, Dawid IB. The homeobox gene *mbx* is involved in eye and tectum development. Dev Biol. 2002;248:107-117.
8. Kawahara A, Dawid IB. Critical role of *biklf* in erythroid cell differentiation in zebrafish. Current Biol. 2001;11:1353-1357.
9. Kodjabachian L, Karavanov AA, Hikasa H, Hukriede NA, Aoki T, Taira M, Dawid IB. A study of Xlim1 function in the Spemann-Mangold organizer. Int J Dev Biol. 2001;45:209-218.
10. Kudoh T, Dawid IB. Role of the *iroquois3* homeobox gene in organizer formation. Proc Natl Acad Sci USA. 2001;98:7852-7857.
11. Kudoh T, Dawid, IB. Zebrafish *mab21l2* is specifically expressed in the presumptive eye and tectum from early somitogenesis onwards. Mech Dev. 2001;109:95-98.
12. Kudoh T, Tsang M, Hukriede NA, Chen X, Dedekian M, Clarke CJ, Kiang A, Schultz S, Epstein JA, Toyama R, Dawid IB. A gene expression screen in zebrafish embryogenesis. Genome Res. 2001;11:1979-1987.
13. Kudoh T, Wilson SW, Dawid IB. Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development. 2002;129:4335-4346.
14. Miyamoto T, Kawahara A, Teufel A, Mukhopadhyay M, Zhao Y, Dawid IB, Westphal H. Mbx, a novel mouse homeobox gene. Dev Genes Evol. 2002;212:104-106.
15. Tsang M, Friesel R, Kudoh T, Dawid IB. Identification of Sef, a novel modulator of FGF signalling. Nat Cell Biol. 2002;4:165-169.

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# PATTERNING NEUROGENESIS IN THE ZEBRAFISH EMBRYO

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The vertebrate nervous system is divided along the anterior-posterior (AP) axis into compartments that form the forebrain, midbrain, hindbrain, and spinal cord. Within each of these specialized compartments, neurons and glia are produced in a stereotyped pattern by neural progenitors. Our laboratory is investigating fundamental patterning mechanisms that are responsible for dividing the nervous system into discrete compartments and ensuring that neurons are made in the appropriate number and location within each compartment. To study molecular mechanisms that are responsible for these early patterning events, we use genetics in zebrafish to identify mutants with aberrant neural patterning. The identification and analysis of *headless* mutants, characterized by the absence of a forebrain and eyes, has allowed us to investigate how a morphogen gradient helps define discrete compartments along the AP axis during early development. Analysis of another mutant, *mind bomb*, which is characterized by the production of too many neurons, has revealed how lateral inhibition, mediated by Notch signalling, helps single out neural progenitors that are permitted to become neurons in the nervous system.

## Cooperative Head Patterning by Two Tcf3 Genes

*Itoh, Chitnis; in collaboration with Dorsky, Moon*

The blastoderm margin is the source of secreted factors that activate gene expression in a dose-dependent manner along the AP axis of the zebrafish gastrula. These factors, including the Wnts and FGFs, are expressed around the blastoderm margin during early gastrulation. They cooperate to establish a gradient of posteriorizing activity in the zebrafish gastrula with its high end around the blastoderm margin and its low end near the animal pole.

Analysis of maternal zygotic (MZ) *headless (hdl)* mutants (Kim C-H *et al.*, 2000) has suggested that posteriorizing factors, in particular Wnts, operate in the context of basal repression provided by hdl, a homolog of T-cell factor-3 (TCF3). Canonical Wnt signalling induces the expression of downstream target genes through the transcriptional activator β-catenin, which associates in the nucleus with Lef/Tcf proteins that bind to DNA-regulatory elements. When β-catenin levels are low, Tcf proteins associate with the co-repressors Groucho and CtBP to maintain target genes in a repressed state.

Loss of repression provided by *hdl* leads to a loss of rostral compartments and expansion of relatively caudal compartments of the neural tube, whose specification depends on posteriorizing factors. We have shown that a second zebrafish *tcf3* homolog, *tcf3b*, limits posteriorization caused by loss of Hdl function. While loss of *hdl* function leads to loss of forebrain and eyes and expansion of the midbrain-hindbrain domain, it leaves the hindbrain relatively unaffected. On the other hand, the additional loss of *tcf3b* function in an MZ *hdl* mutant background leads to further loss of anterior structures and expansion of the hindbrain domain, indicating further posteriorization of the neural tube. Our studies also indicate that, while Hdl and Tcf3b are primarily responsible for repression, a third Lef/Tcf family member, Lef1, may have a primary role in activation of Wnt target genes: injection of *lef1* mRNA does not lead to a recovery of anterior structures in MZ *hdl* mutant embryos as does injection of *hdl* or *tcf3b* mRNA.

By comparing systematic changes in gene expression observed as a consequence of progressive loss of Hdl and Tcf3b function with predictions of computer simulations, we have been able to make specific predictions about the likely shape of the posteriorizing morphogen gradient during early gastrulation. Finally, we have shown that *tcf3b* has a second and unique role in the morphogenesis of rhombomere boundaries, indicating that it controls multiple aspects of brain development.

We are now investigating how the posteriorizing gradient is established and how cells respond to the gradient of posteriorizing factors to define discrete domains of gene expression in the neurectoderm.

### Ubiquitin Ligase Mib Essential for Efficient Activation of Notch Signalling by Delta

*Itoh, Kim, Palardy, Maust, Yeo, Chitnis; in collaboration with Oda, Jiang, Lorick, Wright, Ariza-McNaughton, Weissman, Lewis, Chandrasekharappa*

Neurons are distributed in a simple pattern in the zebrafish neural plate. They are formed in three bilateral longitudinal “proneuronal” domains where cells acquire the potential to become neurons by expressing the proneural gene *neurogenin (ngn1)*. Ngn1 drives the expression of Delta, a membrane-bound ligand that interacts with its receptor Notch in neighboring cells. Activation of Notch by Delta inhibits proneural function. Through this simple feedback loop, each cell within a “proneuronal” domain tends to prevent its neighbors from adopting a similar fate, thus creating a competitive situation. As a consequence of lateral inhibition, a subset of cells eventually emerges as winners and, through auto-regulation, acquires high enough levels of proneural gene expression to permit adoption of a neural fate.

Zebrafish *mind bomb* (*mib*) mutants are characterized by a severe neurogenic phenotype; they also exhibit a wide range of defects in the development of somites, neural crest, and vasculature, which have been interpreted as consequences of deficits in Notch signalling in all these tissues. Though previous studies have suggested that *mib* is likely to encode an essential component of the Notch pathway, the molecular nature of *mib* had remained elusive, and it was not known how it contributes to Notch signalling.

Notch is a one-pass transmembrane receptor that is synthesized as a single peptide. Furin-mediated cleavage of the peptide creates two fragments that are held together in the mature receptor as a heterodimer. The extracellular fragment mediates interactions with the ligand, Delta. An important step in the activation of Notch is the removal of its extracellular fragment. Binding to Delta makes the receptor vulnerable to metalloproteases that cleave Notch at a second site outside its transmembrane domain. In *Drosophila,* it has been shown that the Delta-Notch interaction is accompanied by endocytosis of Delta by the signalling cell, which carries with it the bound Notch extracellular domain. The membrane-bound Notch fragment that remains on the adjacent cell after the cleavage by metalloproteases is a substrate for $\gamma$-secretases. The enzymes cleave the fragment at a third site, within the membrane, which releases an intracellular fragment that functions in a transcriptional activator complex with Su(H)/CBF1/RBP-J to activate Notch target genes that inhibit ngn1 function.

Our analysis has shown that in *mib* mutants reduced lateral inhibition mediated by Notch, permitting too many neural progenitors to differentiate as neurons. Positional cloning of *mib* revealed that it is a novel gene in the Notch pathway, which encodes a RING E3 ubiquitin ligase, an enzyme that plays a central role in ubiquitylation. Ubiquitylation is a multi-step process that results in the addition of a polypeptide, ubiquitin, to a substrate protein. First, a ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent manner; then, a ubiquitin-conjugating enzyme (E2) receives its ubiquitin from an E1. Finally, a ubiquitin ligase (E3), which contains a substrate recognition domain and provides a docking site for an E2, facilitates transfer of ubiquitin from the E2 to its specific substrate. Ubiquitylation was originally recognized for its role in tagging proteins for destruction in proteosomes. More recently, it has been shown that the addition of ubiquitin to proteins can play a pivotal role in changing the behavior or distribution of a protein and can affect a variety of events, including endocytosis.

We have shown that Mib interacts with the intracellular domain of Delta to promote its ubiquitylation and internalization. Cell transplantation studies suggest that *mib* function is essential in signalling cells for efficient activation of Notch in neighboring cells. Our observations provide support for a model in which Mib promotes the trans-endocytosis of the Notch extracellular domain by promoting endocytosis of Delta and, in doing so, facilitates proteolytic events that generate the transcriptionally active Notch intracellular fragment.

## SELECTED PUBLICATIONS

1. Belting HG, Hauptmann G, Meyer D, Abdelilah-Seyfried S, Chitnis A, Eschbach C, Soll I, Thisse C, Thisse B, Artinger KB, Lunde K, Driever W. spiel ohne grenzen/pou2 is required during establishment of the zebrafish midbrain-hindbrain boundary organizer. Development. 2001;128:4165-4176.
2. Itoh M, Chitnis AB. Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. Mech Dev. 2001;102:263-266.
3. Itoh M, Kudoh T, Dedekian M, Kim CH, Chitnis AB. A role for iro1 and iro7 in the establishment of an anteroposterior compartment of the ectoderm adjacent to the midbrain-hindbrain boundary. Development. 2002;129:2317-2327.
4. Kim CH, Oda T, Itoh M, Jiang D, Artinger KB, Chandrasekharappa SC, Driever W, Chitnis AB. Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature. 2000;407:913-916.
5. Kim SH, Shin J, Park HC, Yeo SY, Hong SK, Han S, Rhee M, Kim CH, Chitnis AB, Huh TL. Specification of anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in Zebrafish. Development. 2002;129:4443-4455.
6. Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development. 2001;128:3675-3683.

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During development and differentiation, genes become competent to be expressed or are stably silenced in an epigenetically heritable manner. This selective activation/repression of genes leads to the differentiation of tissue types. Our group is interested in the molecular mechanisms that lead to the heritable transmission of the silenced state. Accordingly, we are studying the mechanism of gene silencing by the Polycomb group genes (PcG) in *Drosophila*. The PcG genes encode a diverse group of proteins known to be important for silencing of homeotic genes during development in organisms as diverse as *Drosophila* and man. Many PcG genes encode chromatin-associated proteins, and it has been proposed that they silence transcription by forming protein complexes that inactivate chromatin. At least three different PcG protein complexes have been characterized that may act by distinct biochemical mechanisms. PcG proteins act through poorly defined *cis*-acting DNA sequences called Polycomb group Response Elements (PREs). PREs are thought to recruit PcG protein complexes to the DNA. Given that there are different types of PcG protein complexes, different types of PREs may also exist. A major focus of our laboratory is to characterize fully the sequences and DNA-binding proteins required for PRE function.

## Polycomb Group Response Elements Require the Activity of Many DNA-Binding Proteins

*Americo, Brown, Grau, Kassis*

We have completed a detailed study of one PRE from the segmentation gene *engrailed*. Within a 139-bp minimal PRE, there are eight protein-binding sites. Our functional data have shown that at least five of the sites are required for PRE activity. One of the proteins that binds to this PRE is the product of the *pleiohomeotic* (*pho*) gene, a known PcG gene. *pho* encodes a *Drosophila* homolog of the mammalian zinc-finger transcription factor Yin Yang 1 (YY1). Another site is the sequence GAGAG, known to bind to two *Drosophila* proteins, GAGA factor and pipsqueak. There are two Pho-binding sites and two GAGAG sequences within the *engrailed* PRE. The proteins that bind to the other four sites have yet to be identified. Isolation of these proteins is a major focus of our laboratory. We are using a yeast one-hybrid screen and biochemical methods to isolate the other DNA-binding proteins involved in this important process. We have recently identified a family of zinc-finger proteins that bind to one of the sequences important for PRE activity. We are currently investigating which of these family members may be involved in Polycomb group repression.

## The Role of *pho-like* in Polycomb Group Repression

*Brown, Kassis; in collaboration with Fritsch, Müller*

Pho-binding sites have been identified in many different PREs, suggesting that Pho is a key component for the recruitment of PcG protein complexes to the DNA. If this were the case, then the phenotype of *pho* mutants should be severe derepression of homeotic genes. Curiously, in *pho* mutants, homeotic genes are only mildly derepressed, but the sequence of the *Drosophila* genome has yielded a possible explanation for this apparent paradox. Another YY1 homolog exists in *Drosophila*. We are studying the function of this gene that we call *pho-like*. We generated mutants in the *pho-like* gene. Flies mutant for *pho-like* are viable, but the females are sterile. Homozygous *pho-like* mothers lay eggs that are fertilized but undergo no nuclear divisions. Thus, Pho-like must be deposited in the egg by the mother for development to occur. Organisms that are double-mutant for *pho* and *pho-like* (from mothers and fathers heterozygous for these two mutations) develop into larvae and have much more severe derepression of homeotic genes than *pho* mutants alone, showing that *pho-like* enhances the *pho* phenotype. In fact, the derepression of homeotic genes seen in imaginal disks from *pho-like*, *pho* double mutants is comparable to that seen in many other PcG mutants. This suggests that Pho and Pho-like play redundant but highly important roles in PcG repression.

Pho-like binds to Pho binding sites *in vitro* and *pho-like, pho* double mutants show more severe misexpression of homeotic genes than the single mutants. But how do these two proteins function? Given that Pho and Pho-like are DNA-proteins, one hypothesis is that they may recruit or anchor PcG protein complexes to the DNA. To address this possible function, we examined the distribution of five PcG proteins on polytene chromosomes from *pho-like, pho* double mutants. These five PcG proteins are not DNA-binding proteins, but they are associated with specific sites on wild-type polytene chromosomes. We reasoned that if Pho and Pho-like are required for anchoring PcG protein complexes to the DNA, then association of PcG chromatin proteins with chromosomal sites might be lost. Our results showed that the PcG proteins Pc, Psc, Scm, E(z), and Ph remain bound to polytene chromosomes at most sites in the absence of Pho and Pho-like. At a few chromosomal locations, however, some of the PcG proteins were no longer present in the absence of Pho and Pho-like. The data suggest the presence of both Pho-like/Pho-dependent and Pho-like/Pho-independent PREs. We submit that, at most genes, both types of PREs exist. Thus, in polytene chromosomes, only a subset of PcG protein complexes may be lost at each genomic location. Alternatively, Pho and Pho-like may act in concert with other PcG proteins but not be required for their association with the DNA.

## Transposon Homing

*Americo, Kassis*

Instead of inserting randomly in the genome like other P-based transposons, P-element–based transposons that contain regulatory DNA from the *Drosophila engrailed* gene (*P[en]*) insert near the endogenous *engrailed* gene about 5 percent of the time. This phenomenon has been called "transposon homing" because the DNA present in the transposon directs it to insert "home" near its parent locus. Only two other cases of transposon homing have been described. DNA from the bithorax complex and the *linotte* gene also cause transposon homing to their parent genes in *Drosophila*. The mechanism of homing is unknown, but it presumably involves the action of proteins bound to the transposon, causing the localization of the transposon to its parent genomic region. It is not known whether similar proteins are responsible for the homing at the three loci. It has been proposed that PREs might be one type of regulatory element that functions in transposon homing. We have tested this proposal by deleting a PRE present in *P[en]*. Deletion of this PRE did not cause a loss of *P[en]* homing, suggesting that PcG proteins may not be involved in transposon homing at *engrailed*.

## SELECTED PUBLICATIONS

1. Americo J, Whiteley M, Brown JL, Fujioka M, Jaynes JB, Kassis JA. A complex array of DNA binding proteins required for pairing-sensitive silencing by a Polycomb-group response element from the Drosophila *engrailed* gene. Genetics. 2002;160:1561-1571.
2. Kassis JA. Pairing-sensitive silencing, Polycomb group response elements, and transposon homing in *Drosophila*. Adv Genet. 2002;46:421-438.

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# REGULATION OF HOMEOTIC GENES IN *DROSOPHILA*

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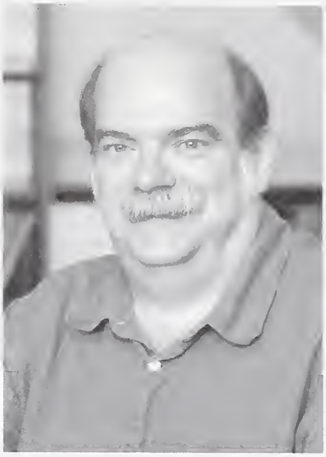

Our goal is to understand the regulation of homeotic gene function in *Drosophila*. The homeotic genes specify segmental identities in *Drosophila* at both the embryonic and adult stages. They encode homeodomain-containing transcription factors that control cell fates by regulating the transcription of downstream target genes. The homeotic genes are expressed in precise spatial patterns that are crucial for the proper determination of segmental identities. Both loss of expression and ectopic expression in the wrong tissues lead to changes in segmental identities. These changes in identity provide a powerful assay to identify the *trans*-acting factors that regulate the homeotic genes and the *cis*-acting sequences through which they act. Both the homeotic genes and the *trans*-acting factors that regulate them are conserved between *Drosophila* and man. In addition to many conserved developmental genes, at least half of the disease- and cancer-causing genes in man are conserved in *Drosophila*, making *Drosophila* an extremely important model system for the study of human development and disease.

## *Cis*-Acting Sequences Required for Transcriptional Regulation of the *Sex combs reduced* Homeotic Gene

*Kennison, Cooper, Southworth*

Assays in transgenes in *Drosophila* have identified *cis*-acting transcriptional regulatory elements from homeotic genes. The assays have identified tissue-specific enhancer elements as well as *cis*-regulatory elements that are required for the maintenance of activation or repression throughout development. While the transgene assays have been important in defining the structure of the *cis*-regulatory elements and identifying *trans*-acting factors that bind to them, their functions within the contexts of the endogenous genes are not yet well understood. We have used a large number of existing chromosomal rearrangements in the *Sex combs reduced* homeotic gene to investigate the functions of the *cis*-acting elements within the endogenous gene. The chromosomal rearrangements identified an imaginal leg enhancer about 35 kb upstream of the *Sex combs reduced* promoter. The imaginal leg enhancer can not only activate transcription of the *Sex combs reduced* promoter that is 35 kb distant, but it can also activate transcription of the *Sex combs reduced* promoter on the homologous chromosome. The *trans*-activation phenomenon was first observed for the homeotic gene *Ultrabithorax* and named transvection. Characterization of the chromosomal rearrangements also revealed that two genetic elements about 70 kb apart in the *Sex combs reduced* gene must be in *cis* to maintain proper repression. When not physically linked to each other, these elements interact with elements on the homologous chromosome and cause derepression of its wild-type *Sex combs* *reduced* gene. To validate our model, we have characterized a transposable element insertional mutation that was isolated 49 years ago and has unusual genetic properties. The transposable element is inserted about 150 kb upstream of the *Sex combs reduced* promoter, and we believe that the unusual genetic properties of the insertion derive from its ability to mimic the endogenous genetic elements required for transcriptional repression. We have identified the transposable element as the *Drosophila Springer* retrotransposon and have used an unlinked genetic suppressor of *Springer* to show that the unusual genetic properties are actually attributable to the *Springer* insertion. We are currently trying to identify the region of the *Springer* retrotransposon that is able to mimic the endogenous *Sex combs reduced* repression elements. We believe that comparisons between the *Springer* sequences and the sequences of the endogenous elements should reveal target sites that interact with the *trans*-acting factors.

### *Trans*-Acting Activators of Homeotic Genes

*Kennison, Green, Cooper, Segal; in collaboration with Eissenberg, Shilatifard, Christensen, Karch, Tamkun, Verrijzer*

Genetic studies have identified the *trithorax* group of genes required for expression or function of homeotic genes. Reduced function of the *trithorax* group genes mimics loss of function of the homeotic genes. Many of the *trithorax* group genes have been shown to be required for the maintenance of transcription of the homeotic genes during development. Many trithorax group proteins are subunits of chromatin-remodeling or transcriptional coactivator complexes. Our section has identified at least two dozen *trithorax* group genes, most of which were not previously identified. Two of the genes that we identified (*skuld* and *kohtalo*) encode subunits of the mediator coactivator complex. The complex is highly conserved between *Drosophila* and man, but only about a third of the subunits are conserved between yeast and man. We have identified several other trithorax group genes that encode subunits of chromatin-remodeling complexes. The *brahma*, *moira*, and *osa* genes encode subunits of the Brahma chromatin-remodeling complex, which is conserved from yeast (the SWI/SNF and RSC complexes) to man (the BRG1 and HBRM complexes). To understand further the function of the Brahma complex, we have been characterizing mutations that interact with mutations in the Brahma complex. As part of these studies, we have recently isolated and characterized mutations in the *Asf1* histone chaperone. We have shown that *Asf1* is important for the structure of heterochromatin and that it interacts both functionally and physically with the Brahma chromatin-remodeling complex. We have also isolated and characterized mutations in *Su(Tpl)*, which encodes the *Drosophila* homolog of the ELL RNA polymerase II transcriptional elongation factor. We have shown that *Su(Tpl)* is required for the transcription of multiple developmental genes, including the *Sex combs reduced* homeotic gene. *Su(Tpl)* is within the first intron of the *Mi-2* gene, which is required for transcriptional repression of the homeotic genes. The regulation of *Su(Tpl)* and *Mi-2* should prove interesting in that both are transcribed from the same DNA strand and in the same cells. We have isolated insertions of a transposon that differentially interfere with the functions of the two genes. The insertional mutations are at the same site in the DNA and differ only in the structure of the transposon.

### *Trans*-Acting Repressors of Homeotic Genes

*Kennison; in collaboration with Müller, Rasmuson-Lestander, McGinnis*

The initial domains of homeotic gene repression are set the by the segmentation proteins, which also divide the embryo into segments. Maintenance of repression requires the proteins encoded by the *Polycomb* group genes. The switch from the initiation to the maintenance of repression involves the recruitment of a chromatin-remodeling complex that includes the *brahma*- and *kismet*-related protein *Mi-2* and a histone deacetylase subunit. In addition, maintenance requires the *Polycomb* complex, which also has a histone deacetylase subunit. In collaboration with J. Müller and A. Rasmuson-Lestander, we have identified and characterized a new *Polycomb* group gene, *Su(z)12*. *Su(z)12* encodes a zinc-finger protein that is required both maternally and zygotically for the maintenance of homeotic gene repression. In collaboration with W. McGinnis, we have also isolated and characterized mutations in the *Deaf-1* gene. The DEAF-1 protein was originally identified as a factor that bound to a *cis*-regulatory enhancer element from the *Deformed* homeotic gene. Although *Deaf-1* is essential for embryonic development, its precise role in transcription is still not understood.

## SELECTED PUBLICATIONS

1. Birve A, Sengupta AK, Beuchle D, Larsson J, Kennison JA, Rasmuson-Lestander A, Müller J. Su(z)12, a novel *Drosophila* Polycomb group gene that is conserved in vertebrates and plants. Development. 2001;128:3371-3379.
2. Eissenberg JC, Ma J, Gerber MA, Christensen A, Kennison JA, Shilatifard A. dELL is an essential RNA polymerase II elongation factor with a general role in development. Proc Natl Acad Sci USA. 2002;99:9894-9899.
3. Kennison JA, Southworth JW. Transvection in Drosophila. Adv Genet. 2002;46:399-420.
4. Moshkin YM, Armstrong JA, Maeda RK, Tamkun JW, Verrijzer CP, Kennison JA, Karch F. Link of histone chaperone ASF1 to chromatin-remodelling machinery. Genes Dev. 2002;16:2621-2626.
5. Schulze S, Sinclair DAR, Silva E, Fitzpatrick KA, Singh M, Lloyd VK, Morin KA, Kim J, Holm DG, Kennison JA, Honda BM. Essential genes in proximal 3L heterochromatin of *Drosophila melanogaster.* Molec Gen Genet. 2001;264:782-789.
6. Southworth JW, Kennison JA. Transvection and silencing of the *Sex combs reduced* homeotic gene of *Drosophila melanogaster.* Genetics. 2002;161:733-746.
7. Veraksa A, Li X, Kennison J, McGinnis W. DEAF-1 function is essential for the early embryonic development of *Drosophila*. Genesis. 2002;33:67-76.

[a] Left NIH in 2000
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# INTEGRATIVE CONTROL OF MACROMOLECULAR SYNTHESIS

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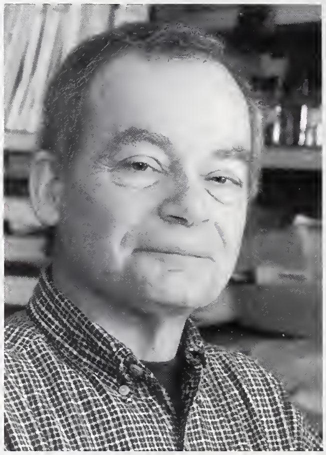

Our goal is to understand how global patterns of bacterial gene expression are coordinated by nutrient availability. Complex networks integrate synthesis of macromolecules and regulate expression of the genomic repertoire, thereby providing a model of regulatory interactions between a cell and its environment. We focus on the roles of two regulatory nucleotides found in most bacteria and recently in plants. The analogs we study are related to GTP and GDP but differ by virtue of a pyrophosphate ester on the ribose 3'-hydroxyl group and are abbreviated as (p)ppGpp. Nutrient limitation elevates basal (p)ppGpp levels, and nutrient sufficiency restores low basal levels. This mechanism operates when bacteria are starved of amino acids, phosphate, nitrogen, or energy sources. Regulatory roles are assigned to (p)ppGpp because eliminating (p)ppGpp can abolish regulation while artificially elevating (p)ppGpp (without nutrient limitation) mimics many regulatory effects of starvation. Responses to (p)ppGpp are an integral element in adaptive responses to starvation, ensuring survival by induction of the stationary phase–specific sigma factor (RpoS) of RNA polymerase and by facilitating its effects on transcription. We therefore wish to understand how nutrient limitation leads to changes in (p)ppGpp levels and how responses to (p)ppGpp operate. Global regulatory mechanisms present in bacteria but not in animals can be exploited to develop new microbial antibiotics.

## Physical Mapping of RelA Binding to Ribosomes

*Glaser, Dahlberg, Murphy, Cashel*

Amino acid starvation is well known to activate synthesis of (p)ppGpp on bacterial ribosomes by the RelA protein; activation occurs because codon-specified uncharged tRNA binds to ribosomal acceptor (A) sites instead of the cognate aminoacylated tRNA for protein synthesis. We wish to understand the molecular details of the interaction between RelA, the ribosome, and the requirement for mRNA-specified uncharged tRNA binding.

Last year, Gad Glaser discovered differences in ribosomal RNA (rRNA) susceptibility to dimethylsulfate (DMS) modification associated with RelA binding. This footprint of specific base modifications was measured for less than half of all rRNA sequences. We also localized the ribosomal binding domain of the RelA protein as the C-terminal region of about 160 amino acids without additional contributions from the remainder of the 744–amino acid protein, which includes the domain catalyzing (p)ppGpp synthesis. This year, we have extended the primer sets to complete the mapping over the entire 16S and 23S rRNA molecules. The more extensive mapping did not appreciably extend our initial estimates of modified domains beyond what was initially discovered, namely, near the CCA end of bound tRNA and the decoding region where A site binds to tRNA anticodons paired with mRNA codons. However, the more precise maps do allow better localization of RelA binding sites to the neighborhood of four (B2b, B3, B5, and B6) of the RNA-RNA helical bridges that, from ribosome structural considerations, are thought to stabilize association of 30S and 50S ribosomal subunits. RelA binding to ribosomes usually increases the sensitivity of rRNA bases to DMS rather than to protect them from chemical modification. This is consistent with the notion that RelA binding opens RNA helices and thereby destabilizes RNA-RNA helical bridges. The structural changes seem so major that one would expect ribosomes with bound RelA not to be competent for peptide bond formation. However, optimization of RelA binding conditions reveals that binding occurs when ribosomes are inactive for protein synthesis. First, the presence or absence of mRNA and tRNA does not alter the binding we observe, although mRNA and tRNA are required for activation of (p)ppGpp synthesis. Second, the ammonium ion concentrations for optimal binding are below the level (100 mM) needed for the "Zamir/Elson" conformational transition, which is known to inactivate peptidyl transferase reversibly. Finally, ribosome-dependent (p)ppGpp synthesis occurs under optimal conditions for RelA binding (in the presence of mRNA and uncharged tRNA) despite a ribosomal conformation with inactive peptidyl transferase. These considerations clearly do not support the long-held view that RelA is associated with functional ribosomes until it is activated by uncharged tRNA binding to A sites, leading to catalysis of a round of (p)ppGpp synthesis which, in turn, leads to dissociation of uncharged tRNA and perhaps also release of the RelA protein. The binding of RelA and the synthesis of (p)ppGpp on a ribosome that is idling for lack of appropriately charged tRNA may instead be mechanistically integrated with failure to form a peptide bond. There is a clear discrepancy between our current observations and what was considered to be true a short time ago.

### Ribosomal Affinity Tag to Immobilize Functional Ribosomes on a Solid Support for Stepwise Studies of Protein Synthesis

*Canna, Murphy, Cashel*

The work just mentioned highlights the need to facilitate functional analysis of the synchronized steps in protein synthesis with respect to binding (or release) of uncharged tRNA as well as RelA protein. The success of existing approaches with immobilized RNA polymerase for addressing a formally analogous problem for RNA, rather than protein, polymerization led us to embark on an attempt to immobilize functional ribosomes as an initial goal. In view of the large number potential of uses of such a construct, it is perhaps surprising that laboratories primarily concerned with protein synthesis have not already attempted the construct.

We have chosen to construct a double-affinity (HisX6 + HA) tag on the 50S ribosomal L1 protein because this protein is among the most surface-exposed ribosomal proteins. In addition, it is distant from the peptidyl transferase catalytic center and distant from RelA binding sites and is reported to have been derivatized on its C-terminus with a GFP tag without compromising ribosomal function as judged by cellular viability. Sequences for the affinity tags were synthesized with (or without) a nine-residue spacer. Scott Canna recombined the sequences into the last codon of *rplA* by using "recombineering" techniques recently developed in Don Court's laboratory (NCI). The ensuing viable chromo-somal constructs were sequenced and genetically mapped by transduction to the appropriate chromosomal locus. Initial studies suggest that nickel-agarose affinity purification of affinity-tagged ribosomes can be achieved whether or not the linker arm is present, although yields are low. Interestingly, Western analyses suggest that anti-HA antibody is reactive only with ribosomes bearing the linker arm, whereas the anti-HisX6 antibody binds regardless of the presence of the linker.

### Regulation of (p)ppGpp by the SpoT Protein

*Gal, Murphy, Cashel; in collaboration with Hogg*

Regulation of (p)ppGpp levels by RelA responds only to amino acid availability. In contrast, regulation of (p)ppGpp by limiting sources of energy, phosphate, or nitrogen is mediated by mechanisms modulating SpoT-catalyzed (p)ppGpp synthetic and/or degradation activities. Interestingly, the SpoT protein does not bind to ribosomes despite broad homology with RelA. We have now constructed a viable, two-residue deletion mutant of SpoT that completely inactivates (p)ppGpp degradation activity without affecting (p)ppGpp synthesis activity. The design of this mutant arose from considerations of Tanis Hogg's crystal structure determination. The mutant strain can now be exploited to assess regulation of synthesis activity unobscured by effects on degradation. Conserved domain searches from Eugene Koonin's laboratory (NCBI) have revealed potential clues to SpoT regulation. The SpoT protein has a TGS domain (threonyl-tRNA synthetase and GTPase), followed by an ACT domain (shared by aspartokinase, chorismate mutase, and TyrA) that is deduced to be capable of binding low molecular–weight ligands. Jozsef Gal has used appropriate portions of the SpoT protein as bait to screen an *E. coli* library with a commercial bacterial two-hybrid system. Multiple isolates of two genes have passed screening tests for false positives: one is known to be involved in iron uptake while the function of the other is unknown. We are currently mapping the interacting regions of the candidate proteins and asking whether chromosomal deletions of the genes have a SpoT-dependent (p)ppGpp regulatory phenotype.

## SELECTED PUBLICATIONS

1. Brown L, Gentry D, Elliott T, Cashel M. DksA affects ppGpp induction of RpoS at a translational level. J Bacteriol. 2002;184:4455-4465.
2. Mechold U, Murphy H, Brown L, Cashel M. Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel*Seq* the Rel/Spo enzyme from *Streptococcus equisimilis.* J Bacteriol. 2002:184:2878-2888.

## COLLABORATORS

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# MOLECULAR GENETICS OF MAMMLIAN RETROVIRUS REPLICATION

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The goal of our research is to define the molecular mechanisms involved in the replication of HIV and related retroviruses. These studies are critical for developing new strategies to combat the AIDS epidemic, which has continued to spread to all parts of the world and is a serious threat to the health and well-being of both children and adults. To address these issues, we have developed reconstituted model systems to investigate the individual steps in HIV-1 reverse transcription, a major target of HIV therapy. Much of our work focuses on the viral nucleocapsid protein (NC), which promotes highly efficient and specific viral DNA synthesis. NC is a nucleic acid chaperone, which means that it can facilitate nucleic acid conformational rearrangements that lead to formation of the most thermodynamically stable structure. This activity is essential for viral DNA synthesis. In other studies, our efforts are directed toward understanding the function of the viral capsid protein in HIV-1 assembly and early post-entry events during the course of virus replication *in vivo*.

## Role of Nucleocapsid Protein in HIV-1 Strand Transfer

*Guo, Heilman-Miller, Levin; in collaboration with Gorelick, Henderson, Musier-Forsyth*

HIV-1 NC is a small basic protein with two zinc fingers, each containing the invariant CCHC zinc-coordinating residues. The remaining amino acids within each finger are not identical. NC function in virus replication depends on its dynamic interaction with nucleic acids, and the protein plays a critical role in the two strand-transfer steps that occur during viral DNA synthesis. We have shown that, during minus-strand transfer, the nucleic acid chaperone activity of NC destabilizes the highly structured complementary TAR stem-loop (TAR DNA) at the 3' end of (-) strong-stop DNA and inhibits TAR-induced self-priming, a dead-end reaction that competes with strand transfer. In our recent work, we have focused on the functional significance of the two zinc fingers for NC nucleic acid chaperone activity and on measurement of NC-induced conformational changes in (-) strong-stop DNA, which form the basis for inhibition of self-priming.

To address the issue of zinc finger function, we have used a mutational approach. Initially, we investigated the effects of an NC mutation that eliminates zinc coordination by changing the two CCHC motifs to SSHS. Our results provided the first direct evidence that zinc coordination is required for inhibition of self-priming and efficient minus-strand transfer. More recent findings demonstrate that, for optimal minus-strand transfer, CCHH or CCCC (zinc-binding motifs found in cellular proteins) cannot replace the CCHC motifs and that the amino acid context within each finger must be preserved. Context changes also reduce the ability of NC to facilitate tRNA primer removal, a step that is required for plus-strand transfer. In addition, we have found that the N-terminal zinc finger is a more critical determinant of NC nucleic acid chaperone activity than the C-terminal finger. Interestingly, our *in vitro* results correlate with earlier *in vivo* replication data.

To gain a better understanding of NC-mediated inhibition of self-priming, we are collaborating with Karin Musier-Forsyth and colleagues who have developed a fluorescence resonance energy transfer (FRET) assay that makes it possible to monitor conformational changes directly in TAR DNA when NC is present. The results show that when NC binds to TAR DNA alone, there is only a modest shift toward less-folded states. In the presence of acceptor RNA, NC binding to TAR DNA results in a shift of the majority of molecules to the unfolded state. These data agree with biochemical observations we made by using a modified strand transfer system containing preformed (-) strong-stop DNA and acceptor RNA. Thus, in the absence of acceptor RNA, NC has little effect on self-priming, whereas when acceptor RNA is present, NC stimulates strand transfer, and self-priming is dramatically reduced. Work is now under way to define the structural requirements for NC interaction with the strand transfer nucleic acid intermediates. A series of synthetic (-) strong-stop DNA and acceptor RNA truncation mutants have been constructed and are under investigation by *in vitro* assay of minus-strand transfer and self-priming, enzymatic structure probing, and analysis of secondary structure using RNA and DNA structure prediction algorithms.

### Nucleic Acid and Protein Requirements for Initiation of HIV-1 Reverse Transcription

*Iwatani, Levin; in collaboration with Musier-Forsyth*

We have begun an investigation of the initiation step in HIV-1 reverse transcription. The event is primed by a host tRNA, $tRNA_3^{Lys}$, that is annealed to the 18-nt priming binding site (PBS) near the 5' terminus of the viral RNA genome; extension of the primer leads to synthesis of a short DNA product known as (-) strong-stop DNA. Earlier studies showed that *cis*-acting genomic RNA sequences upstream of the PBS are important for efficient (-) strong-stop DNA synthesis, but a possible role of sequences downstream of the PBS has not yet been established. As a first approach, we constructed a series of 14 RNA templates with varying lengths of downstream sequences. (-) Strong-stop DNA synthesis catalyzed by RT was first measured under conditions wherein the template was heat-annealed to each of three primers: $tRNA_3^{Lys}$; and 18-nt RNA (R18) or DNA (D18) oligonucleotides, each bearing the sequence of the 3' 18 nt of the tRNA. Interestingly, a minimum of 24 bases downstream of the PBS was required when the RNA primers, but not the D18 primer, were used. To investigate this phenomenon further, we also tested 18-nt DNA-RNA chimeric primers in our assay; 5'-R9D9 and 5'-R17D1 behaved like the D18 and R18 primers, respectively, while 5'-D9R9 exhibited "RNA-like" primer activity. Thermal stability (as determined by melting studies) and circular dichroism spectra of 18-nt primer:PBS duplexes were correlated with the biochemical data. The results point to the importance of helical conformation and stability as determinants of priming activity. Surprisingly, when NC was present, the additional 24 bases downstream of the PBS were dispensable for synthesis primed by tRNA but not for synthesis primed by the R18 primer.

Taken together, our findings suggest that, in the absence of NC, sequences downstream of the PBS are required to induce a conformation of the initiation complex that results in more efficient (-) strong-stop DNA when an RNA primer is used. We propose that NC abrogates this requirement by facilitating stable formation of extended interactions between the full-length tRNA and the RNA template, which are not possible with an 18-nt RNA.

### Functional Analysis of HIV-2 Reverse Transcriptase Activities

*Post, Guo, Levin; in collaboration with Powell, Le Grice, Hizi*

The number of HIV-2 replication studies is relatively small as compared with the enormous literature on HIV-1 replication. Both viruses show similar genome organization, protein composition, and mode of transmission but differ in time of onset of AIDS and geographic pattern of infection (HIV-2 infection occurs primarily in West Africa and parts of Asia, where it is a significant public health issue). In addition, drugs that inhibit HIV-1 replication are not necessarily effective against HIV-2, posing a major problem for clinical treatment of HIV-2–infected individuals. We have conducted a systematic evaluation of the functional activities of HIV-2 RT, a potential target for antiviral therapy, by using assays that mimic specific steps in reverse transcription. We also performed parallel studies with HIV-1 RT.

In general, under standard assay conditions, the RNA- and DNA-dependent polymerase activities as well as the 5'-directed RNase H activities of the two enzymes are comparable (i.e., differences lie within an approximately two-fold range). In contrast, when the concentration of RT is reduced 100- or 1,000-fold, more striking differences are observed and the activity of HIV-2 RT is significantly reduced. Furthermore, under the conditions used for an assay that models tRNA primer removal, the ability of HIV-2 RT to catalyze secondary RNase H cleavage is also impaired. Moreover, initiation of plus-strand DNA synthesis is much less efficient with HIV-2 RT compared with HIV-1 RT unless the salt and magnesium ion concentrations are lowered to 5 mM and 3 mM, respectively. We have previously shown that plus-strand initiation depends on nucleic acid contacts with "primer grip" residues in the palm subdomain of the p66 RT subunit. We now propose that the reduced activity of HIV-2 RT in the plus-strand priming reaction may reflect architectural differences in the primer grip regions of the two enzymes. Taken together, our findings should be useful for development of specific high-throughput screening assays of potential HIV-2 inhibitory agents.

### Function of HIV-1 Capsid Protein in Virus Assembly and Early Post-Entry Events

*Tang, Levin; in collaboration with Freed*

Our laboratory has been investigating the role of the HIV-1 capsid protein (CA) in early post-entry events, a stage in the infectious process that is still not completely understood. Structural studies of CA showed that a group of conserved hydrophobic residues faces the interior of the coiled coil–like structure within the N-terminal domain, and it was suggested that these residues might be important for CA structure and function. Recently, as a result of using genetic, molecular, and ultrastructural approaches, we reported the unusual phenotype associated with single alanine substitution mutations in these residues. Mutant virions are not infectious and lack a cone-shaped core. Moreover, despite their having a functional reverse transcriptase, the mutants are blocked in viral DNA synthesis in infected cells, indicating a defect in an early post-entry event preceding reverse transcription.

Current efforts focus on elucidating the mechanism by which these CA mutations disrupt virus infectivity. The work has uncovered several novel properties of the mutants. We have observed that the mutations block the incorporation of host cyclophilin A (a peptidyl-prolyl *cis-trans* isomerase required for virus replication) into virions. This finding was unexpected, given that the mutated residues are distant from the cyclophilin A binding loop in CA. The results indicate that the mutations induce conformational changes in CA that have global effects on CA structure and function. To investigate the possibility that the mutants might also be compromised in an early post-entry step, we modeled disassembly (i.e., viral uncoating) *in vitro* by generating viral cores following treatment of virus particles with mild detergent. In contrast to wild-type, less RT is associated with mutant cores. What is most striking, however, is the extraordinarily high retention of CA in mutant cores, indicating that mutant cores are unusually stable. Such stability would interfere with proper disassembly and, together with the reduced level of RT in mutant cores, would account for the failure of these mutants to synthesize viral DNA following virus entry into cells.

Our results reveal for the first time the crucial role of the N-terminal hydrophobic core in HIV-1 replication. The critical importance of these residues for maintaining CA structure and function indicates that the hydrophobic motif represents a potential new target for antiviral drugs and other therapeutic approaches for combating the AIDS epidemic.

## SELECTED PUBLICATIONS

1. Guo J, Wu T, Anderson J, Kane BF, Johnson DJ, Gorelick RJ, Henderson LE, Levin JG. The zinc finger structures in the human immunodeficiency virus type 1 nucleocapsid protein facilitate efficient minus- and plus-strand transfer. J Virol. 2000;74:8980-8988.
2. Guo J, Wu T, Kane BF, Johnson DG, Henderson LE, Gorelick RJ, Levin JG. Subtle alterations of the native zinc finger structures have dramatic effects on the nucleic acid chaperone activity of human immunodeficiency virus type 1 nucleocapsid protein. J Virol. 2002;76:4370-4378.
3. Johnson PE, Turner RB, Wu ZR, Hairston L, Guo J, Levin JG, Summers MF. A mechanism for plus-strand transfer enhancement by the HIV-1 nucleocapsid protein during reverse transcription. Biochemistry. 2000;39:9084-9091.
4. Tang S, Murakami T, Agresta BE, Campbell S, Freed EO, Levin JG. Human immunodeficiency virus type 1 N-terminal capsid mutants that exhibit aberrant core morphology and are blocked in initiation of reverse transcription in infected cells. J Virol. 2001;75:9357-9366.

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# PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR GENETIC EVENTS OF RECOGNITION AND RESOLUTION OF RNA-DNA HYBRIDS

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Our research is directed toward understanding processes involved in cellular DNA replication and the relationship of HIV replication to these cellular events and using such information for therapeutic purposes. We are examining the formation and resolution of RNA-DNA hybrids formed during DNA replication or transcription. Ribonucleases H are important enzymes that participate in the removal of RNA from the RNA-DNA hybrids and are intimately involved in DNA replication in cells and, during HIV replication, in the conversion of the RNA genome of this virus to DNA. Using a similar protein architecture, RNases H of cells and HIV share common enzymatic mechanisms of RNA cleavage. Drugs to alter levels of specific disease-related genes are under development to take advantage of RNases H within the cell. Regulated expression of RNases H could enhance the efficacy of the drugs. Molecular genetic, biochemical, and mouse animal models are central to our efforts.

## RNase H1 Requirement for Embryogenesis in Mice

*Cerritelli, Crouch; in collaboration with Love*

Mouse and human RNases H1 have an N-terminal stretch of amino acids that is absent from RNases H1 of simple eukaryotes (e.g., yeasts) and has the properties of a mitochondrial localization sequence. Using transient transfection assays, we demonstrated mitochondrial targeting by the 26 amino acid leader of the mouse RNase H1. We made an *Rnaseh1* knockout to test for the effects on mitochondrial function *in vivo*. We found that growth is arrested in *Rnaseh1*$^{-/-}$ mice *in utero* at day E8.5, that the mice have defective respiratory function, and that they exhibit massive apoptotic cell death; the latter two effects are both direct results of a failure in mitochondrial DNA replication. Mitochondrial DNA replication occurs by two mechanisms, and our results suggest that RNase H1 is required in one or both. Mammalian oocytes contain numerous mitochondria, which, upon fertilization, become distributed without amplification to cells of the developing embryos. As a consequence, mitochondrial DNA replication is delayed until midgestation. We suggest that, during development, the two modes of mitochondrial DNA replication are likely to be activated sequentially and that RNase H1 is required for the earliest system, which is established during mouse embryogenesis.

### Mechanism of Action of Mouse and Human RNases H1

*Gorshkova, Gaidamkov, Cerritelli, Crouch: in collaboration with Schuck*

A few years ago, we discovered that eukaryotic RNases H1have an RNase H domain similar in size, amino acid sequence, and structure to that we reported for *E. coli* RNase HI. Eukaryotic RNases H1 have an additional domain connected to the N-terminus of the RNase H domain, which can bind to duplex RNAs even in the absence of the RNase H domain. We have now shown that this "extra" dsRNA-binding domain aids in dimerization of the full-length protein when an RNA-DNA substrate is present. Moreover, the presence of the dsRNA-binding region transforms the RNase H activity from random to processive (i.e., the enzyme remains bound to the substrate until as much of the substrate as possible is degraded). Most RNA-DNA hybrids used for priming DNA synthesis are relatively short and would not seem to require processivity. We know that one exception is the RNA-DNA formed during mitochondrial DNA replication. Thus, the findings described above in the previous section fit well with the properties of RNase H1 we have uncovered.

### Eukaryotic RNase H2 Activation

*Jeong, Crouch*

Bacterial and archael RNases HII are active as a single polypeptide chain, and several key residues have been shown to be a part of the active site. The eukaryotic RNase H2 enzyme is considerably larger than RNases HII, yet the orthologous eukaryotic polypeptide is similar in size and sequence to the smaller RNases HII. We have expressed the RNase H2 polypeptide encoded by human, mouse, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* in *E. coli* and uniformly found the protein to have no RNase H enzymatic activity. It seems that eukaryotic RNases H2 require some modification and/or more than one subunit for RNase H2 activity. From *S. cerevisiae* cells, we have affinity-purified the RNase H2 polypeptide of *S. cerevisiae* and found that two proteins co-purify in a complex. The identities of these proteins have been determined by using mass spectrometry. RNase H2 activity is absent from *S. cerevisiae* strains in which either of the two genes encoding these proteins has been deleted, suggesting that both proteins may be components of the active enzyme. The two proteins have been reported to interact with each other, but no evidence has linked them to RNase H2. However, they have been connected with some of the protein kinases involved in cell cycle regulation. Earlier work in our group demonstrated cell cycle regulation of *S. cerevisiae* RNase H2.

## SELECTED PUBLICATIONS

1. Arudchandran A, Cerritelli SM, Bowen NJ, Chen X, Krause MW, Crouch RJ. Multiple ribonuclease H-encoding genes in the *Caenorhabditis elegans* genome contrasts with the two typical ribonuclease H-encoding genes in the human genome. Mol Biol Evol. 2002;19:1910-1919.
2. Gorshkova II, Rausch JW, Le Grice SFJ, Crouch, RJ. HIV-1 reverse transcriptase interaction with model RNA-DNA duplexes. Anal Biochem. 2001;29:198-206.
3. Lee C-G, Kinoshita K, Arudchandran A, Cerritelli SM, Crouch RJ, Honjo T. Germline transcription positively correlates with class switch recombination efficiency. J Exp Med. 2001;194:365-373.
4. Puertollano R, Aguilar RC, Gorshkova II, Crouch RJ, Bonifacino JS. Sorting of mannose 6-phosphate receptors mediated by the GGAs. Science. 2001;282:1712-1716.

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# CONTROL OF ECTODERMAL DEVELOPMENT IN *XENOPUS*

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We focus on discovering mechanisms that regulate the development of tissues arising from the ectodermal region of vertebrate embryos. Our experimental model system is the frog *Xenopus laevis*. Promoter analysis, subtractive hybridization and microarray analysis, and *in situ* hybridization studies have identified genes encoding factors that both respond to secreted embryonic signals, such as bone morphogenetic proteins (BMPs), and mediate localized differentiation into ectodermal derivatives such as neural plate, neural crest, and epidermis. More specifically, the studies have identified two basic categories of regulatory factors: negatively acting Distal-less-like homeobox factors Dlx3, Dlx5, and Dlx6 and the related homeobox factor Msx1; and the positively acting transcription factor AP-2α. The Msx/Dlx genes respond differentially to a graded signal generated by antagonistic interactions between secreted BMPs and inducers originating in the Spemann Organizer region. This sets up different territories in the ectoderm in which different "codes" of Msx/Dlx gene expression appear to define specific tissue types. AP2α also is regulated by BMP signaling. In the epidermis, AP2α is necessary for expression of structural genes. A combination of BMPs and secreted factors in the Wnt class leads to a rise in the level of AP2α, which is essential in the induction of neural crest.

## AP2α in Epidermal Development

*Luo, Thomas, Sargent; in collaboration with Weeks*

The group's earlier work suggested that AP2α might be important in controlling the expression of certain epidermal genes in *Xenopus*. Two different types of experiment have now confirmed and considerably extended our previous work. First, AP2α was artificially restored to ectoderm from embryos in which BMP signaling was blocked by over-expressing a BMP antagonist (chordin). BMP signaling is required for epidermal development. This AP2 "rescue" experiment resulted in the reactivation of epidermal gene expression, which was inhibited by the BMP antagonist. We obtained the same result even when we inhibited endogenous protein synthesis with cycloheximide, indicating that AP2α is immediately upstream from epidermal genes and is a mediator of BMP signaling at the transcriptional level. A second experiment used antisense oligonucleotides designed to mediate the destruction of endogenous AP2α mRNA. The loss-of-function for AP2α resulted in suppression of all epidermal gene expression and activation of neural genes (as usually happens in *Xenopus* when epidermis is inhibited). Gastrulation movements were also disturbed in such embryos. Therefore, AP2α is required for epidermal cell differentiation, including both the expression of molecular phenotypic markers and the rearrangement of epidermal cells that accompanies gastrulation in the frog.

### Dlx3 and AP2α in the Neural Crest

*Luo, Sargent; in collaboration with Saint-Jeannet*

It is generally accepted that some form of spatial gradient of BMP signaling provides an important cue for localized differentiation in early vertebrate embryos. However, little is known about how such spatial information is translated into region-specific gene expression. We have made some important progress in expanding our knowledge by showing that Msx1, Dlx3, and Dlx5 are specifically excluded from cement gland, neural crest, and neural plate, respectively, probably as the result of differential responsiveness to graded BMP signaling within the ectoderm. When misexpressed by RNA injection, or in "animal cap" assays, these factors selectively inhibit the differentiation of cell types from which they are normally excluded. The inhibition of neural crest development by Dlx3 misexpression is consistent with our earlier observation that Dlx3 can act as an antagonist of Wnt/β–catenin signaling in frog embryo: Wnt signaling is required for neural crest induction. Thus, Dlx3 may function both to interpret intermediate BMP signal strength and to confine the response to Wnt signaling, spatially limiting the neural crest territory.

AP2α has been used as a marker for cranial neural crest for some time, and AP2α-null mice have major craniofacial abnormalities, suggesting a role for this factor in neural crest development, at some level. Using the same tools that revealed the need for AP2α function in the epidermis, we have shown a similarly essential role for AP2α in the initial specification of neural crest in *Xenopus*. We are currently testing the theory that positive control by AP2α and negative control by repressors including Dlx3 work together to specify this important tissue type.

### Regulatory Networks

*Luo, Lepperdinger, Khadka, Zhang, Sargent; in collaboration with Chandhoke, Grant, Christensen, Fryxell*

In this project, we are using Dlx and AP2α genes as starting points to work out regulatory "wiring diagrams" for the induction and differentiation of epidermis and neural crest. The guiding hypothesis is that the control of these developmental programs is largely transcriptional in nature but depends on both direct interaction of regulatory proteins with target sequences in genomic DNA and protein-protein interactions with other control factors. We are thus taking two approaches to identifying genes that interact with Dlx3/5 and AP2α: target gene identification by microarray analysis and two-hybrid screens for interacting proteins.

In an initial study, we generated a subtracted library enriched in epidermis-specific genes. From this population, we performed 2,000 cDNA sequence reads, yielding about 1,000 unique expressed sequence tags (ESTs)s. Our collaborators at George Mason University amplified and spotted the ESTs. We then hybridized the arrays with probes derived from animal caps of embryos injected with BMP antagonists with the aim of identifying novel BMP-dependent genes that might be involved in regulating the formation of the epidermis during gastrulation. While the process led to the identification of several interesting ESTs and a number of previously characterized genes, it did not suggest any direct targets of AP2α or indicate that connecting the genes' function with that of AP2α or other epidermal factors would be a straightforward proposition. A somewhat more focused microarray project is now under way. We are generating a subtracted and normalized cDNA library that should be enriched in early-expressed genes specific to the neural crest. Approximately 3,000 cDNA clones will be amplified and spotted onto glass microarrays. Probes will then be derived from animal cap ectoderm treated with various inducers and repressors of neural crest, including reagents specific for AP2α. At a minimum, we hope to identify novel neural crest marker genes that would augment the rather small collection currently available. Ideally, this approach will also identify candidate targets for the action of AP2α as a transcriptional activator in neural crest precursor cells. Subsequent gain and loss of function experiments can then be used to determine functional significance while transcriptional assays in embryos and animals caps can be employed to analyze the interactions between target DNA elements and AP2α protein.

To search for proteins that interact physically with either Dlx3/5 or AP2α, we are using a bacterial "two hybrid" system with both commercially available cDNA libraries and specialized libraries constructed in our laboratory. We hope that this prokaryotic system will circumvent the cell cycle interference toxicity we previously encountered with Dlx3 in a yeast two-hybrid system. Any candidate interaction factors will be subjected to whole-mount *in situ* hybridization analysis and ultimately to functional studies using antisense or dominant negative overexpression strategies.

## SELECTED PUBLICATIONS

1. Beanan MJ, Feledy JA, Sargent TD. Regulation of early expression of Dlx3, a Xenopus anti-neural factor, by β-catenin signaling. Mech Dev. 2000;91:227-235.
2. Luo T, Matsu-Takasaki M, Lim JH, Sargent TD. Differential regulation of Dlx gene expression by a BMP morphogenetic gradient. Int J Dev Biol. 2001;45:681-684.
3. Luo T, Matsuo-Takasaki M, Sargent TD. Distinct roles for Distal-less genes Dlx3 and Dlx5 in regulating ectodermal development in Xenopus. Mol Reprod Dev. 2001;60:331-337.
4. Luo T, Matsuo-Takasaki M, Thomas ML, Weeks DL, Sargent TD. Transcription factor AP-2 is an essential and direct regulator of epidermal development in Xenopus. Dev Biol. 2002;245:136-144.

## COLLABORATORS

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[a] Left May 2002
[b] Arrived August 2002

# ORGANOGENESIS OF THE ZEBRAFISH VASCULATURE

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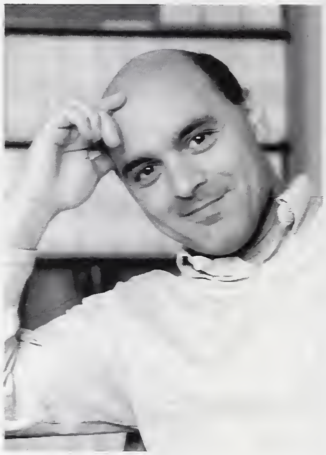

Our objective is to understand the cellular and molecular mechanisms responsible for the specification, patterning, and differentiation of internal organs during development, specifically, how the elaborate network of blood vessels arises during vertebrate embryogenesis. Many of our insights into mechanisms of blood vessel formation have come from developmental studies. Given the potential shown by antiangiogenic therapies for combating cancer, blood vessels have also become a subject of great clinical interest in recent years.

The zebrafish, a small tropical freshwater fish, possesses a unique combination of features that make it particularly well suited for studying blood vessels. The fish is a genetically tractable vertebrate with a physically accessible, optically clear embryo. These features facilitate the study of vascular development by permitting observation of every vessel in a living animal and simple, rapid screening for even subtle vascular-specific mutants. Major aims of the laboratory include developing new experimental tools and resources to enhance the zebrafish as an experimental model for studying vascular embryogenesis; studying the molecular basis for arterial-venous differentiation; studying the role of neuronal guidance factors in vascular guidance and patterning; and performing forward genetic analysis of blood vessel development by using vascular-specific mutants.

## Tools for Experimental Analysis of Vascular Development in the Zebrafish

*Isogai, Lawson, Subramanian*

To exploit fully the advantages of the zebrafish, an important aim has been to develop new experimental tools for studying blood vessel formation in this organism. Previously, we devised a microangiographic method for imaging patent blood vessels in the zebrafish and used it to compile a comprehensive, staged atlas of the vascular anatomy of the developing fish (Isogai *et al.*, 2001; http://eclipse.nichd.nih.gov/nichd/lmg/redirect.html). We have also generated several different transgenic zebrafish lines expressing green fluorescent protein (GFP) in vascular endothelial cells (VEC), making it possible for us to visualize the blood vessel formation in intact, living embryos (Motoike *et al.*, 2000; Lawson and Weinstein, 2002a). We have developed methodologies for long-term multiphoton confocal time-lapse imaging of the dynamics of blood vessel formation in these transgenic zebrafish and have used the methodologies to examine the morphogenesis of intersegmental (Isogai *et al.*, 2002, submitted) and cranial (Lawson and Weinstein, 2002a) blood vessels. Our findings highlight the extremely dynamic, unexpectedly growth cone–

like behavior of growing angiogenic blood vessels. These findings are reinforced by our analysis of the role of growth cone guidance factors in vascular patterning (see below). We have continued our efforts to identify new genes expressed in the zebrafish vasculature, uncovering zebrafish orthologs of angiopoietins-1 and -2 (Pham *et al.*, 2001), VE-cadherin, and myocardin, to name a few. Finally, we have performed a preliminary characterization of the morphology and time course of vascular smooth muscle cell (VSMC) acquisition and vessel wall formation in the zebrafish. Our results show that the vascular wall forms in similar fashion in zebrafish and other vertebrates, providing a foundation for further experimental study of VSMC and VSMC-VEC interaction in the zebrafish.

### Molecular Dissection of Arterial-Venous Development

*Kamei, Lawson, Vogel*

We have uncovered a molecular pathway regulating the acquisition of arterial-venous identity. Although the fundamental distinction between these two types of blood vessel has been appreciated for thousands of years, the fact that arterial and venous endothelial cells have distinct molecular and functional identities has become apparent only very recently, and the mechanisms responsible for establishing this identity have not yet been elucidated. We have now shown that sonic hedgehog (SHH), vascular endothelial growth factor (VEGF), and notch signaling act in series to determine arterial-venous identity (Lawson *et al.*, 2001; Lawson *et al.*, 2002). We were able to do this by using different combinations of drug treatments, mutants, morpholinos, and mRNA injections to activate or repress the activity of each of these signals *in vivo,* either alone or in combination. Our surprising findings regarding the novel role of VEGF in arterial specification have been confirmed by a number of very recent publications from other laboratories that have described a similar activity for VEGF in mice. We are now exploring additional factors that might participate in the pathway we have uncovered, including the zebrafish *DeltaC* gene and a vascular-specific phosopholipase C-gamma gene we recently uncovered in a mutagenic screen (see below).

### Analysis of the Roles of Neuronal Guidance Factors in Vascular Patterning

*Berk, Torres-Vazquez, in collaboration with Kuwada, Li, Chien, Epstein*

We have recently become interested in the role that well-known neuronal guidance factors might play in vascular guidance and vascular patterning. In particular, we are studying the roles of the Slit-Robo and Semaphorin-Plexin ligand-receptor pairs in vascular patterning. Both of these ligand-receptor pairs are known to play important roles in axonal pathfinding, mostly via repulsive interactions between Slit or Semaphorin ligands and Robo- or Plexin-bearing neuronal growth cones. In collaboration with researchers studying related murine genes, we have uncovered novel Robo and Plexin receptors expressed in zebrafish blood vessels and have examined their functional roles. The Robo4 receptor is expressed in both neuronal and vascular tissues in zebrafish. Vascular-specific expression of a dominant-negative truncated form of either the murine or zebrafish *Robo4* genes in zebrafish embryos (via transient transgenesis) results in premature vascular sprouting, consistent with the pro-migratory activity of this construct in murine cell culture (Park *et al.*, 2002, submitted). The *PlexinD* gene is expressed in an entirely vascular-specific fashion in both mice and zebrafish. Targeted knock-down of zebrafish *PlexinD* using antisense morpholine oligonucleotide injection results in aberrant pathfinding of trunk intersegmental vessels and other vessels. Further analysis of the functional roles and activities of both of these receptors and their ligands is in progress. Our work suggests that the mechanisms of axonal and vascular guidance and patterning have a great deal in common, including specific molecular guidance factors.

### Isolation and Analysis of Vascular-Specific Mutants

*Berk, Diamond, Kamei, Lawson, Mugford, Pham, Roman, Torres-Vazquez; in collaboration with Dawid, Lechleider, Liu, Moon*

Genetic dissection of vascular development and the molecular pathways that regulate it is an important ongoing goal of the group. We employ unbiased, forward genetic mutational screening approaches to identify and then perform phenotypic and molecular characterizations of zebrafish mutants that affect the formation of the developing vasculature. We have already positionally cloned the defective genes from a number of previously identified vascular patterning mutants. Resulting from defects in a zebrafish ortholog of the TGF-beta superfamily receptor *acvrl1* (Roman *et al.*, 2002), *violet beauregarde* mutants have abnormal cranial vascular patterning and circulation. Defects in human *acvrl1* cause hereditary hemorrhagic telangiectasia type 2 (HHT), an inherited vascular disorder characterized by arterial-venous malformations with a high incidence of hemorrhage and stroke. Another mutation, *kurzschluss*, has defects in the posterior aortic arches caused by defects in *smap1*, a chaperonin expressed in the mesenchyme surrounding the arch vessels that may be involved in regulating myosin assembly. We have also performed new screens for vascular-specific mutants by using transgenic zebrafish expressing green fluorescent protein (GPF) in blood vessels. We identified 11 new vascular mutants in a pilot screen of haploid progeny of NEU-mutagenized animals that was performed with the Dawid and Liu laboratories (members of these laboratories independently screened for hematopoietic and neuronal defects). Two of the new mutants have defects in a phospholipase C-gamma *(plcg)* gene expressed specifically in the vasculature. PLCGs act downstream of receptor tyrosine kinase signaling, including that induced by vascular endothelial growth factor (VEGF). Like animals in which VEGF itself is directly targeted, *plcg* mutants display defects in arterial endothelial differentiation. Molecular and phenotypic characterization of other mutants from this pilot screen is in progress. We have recently initiated a larger-scale F3 diploid screen of NEU-mutagenized animals (to be carried out with members of the Dawid and Chitnis laboratories) to screen for mutants affecting both intersegmental vessel formation and later vascular patterning events that cannot be examined in haploid animals. Our experience suggests that these ongoing mutant screenings should continue to yield a rich harvest of novel vascular-specific mutants and bring to light new pathways regulating the specification, differentiation, and patterning of the developing vertebrate vasculature.

## SELECTED PUBLICATIONS

1. Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. Dev Biol. 2001;230:278-301.
2. Lawson ND, Weinstein BM. Arteries and veins–making a difference with zebrafish. Nat Rev Genet. 2002b;3:674-682.
3. Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol. 2002a;248:307-318.
4. Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis A, Campos-Ortega JA, Weinstein BM. Notch signaling is required for arterial-venous differentiation during vascular development. Development. 2001;128:3675-3683.
5. Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch signaling pathway during arterial endothelial cell differentiation. Dev Cell. 2002;3:127-136.
6. Lyons SE, Lawson ND, Lei L, Bennett PE, Weinstein BM, Liu PP. A nonsense mutation in *gata1* causes the bloodless phenotype in *vlad tepes*. Proc Natl Acad Sci USA. 2002;99:5454-5459.
7. Motoike T, Loughna S, Liao W, Roman BL, Perens E, Chau TC, Richardson CD, Kawate T, Kuno J, Weinstein BM, Stainier DYR, Sato TN. Universal GFP reporter for the study of vascular development. Genesis. 2000; 28:75-81.
8. Pham VN, Roman, BL, Weinstein BM. Isolation and expression analysis of three zebrafish angiopoietin genes. Dev Dyn. 2001;221:470-474.
9. Roman BL, Pham VN, Childs S, Kulik M, Lawson ND, Lekven AC, Neubaum D, Fishman MC, Lechleider RJ, Moon RT, Weinstein BM. Disruption of *acvrl1* increases endothelial cell number in zebrafsh cranial vessels. Development. 2002;129:3009-3019.

10. Sehnert AJ, Huq A, Weinstein BM, Walker C, Fishman MC, Stainier DYR. Cardiac troponin T plays essential roles in sarcomere assembly and contraction. Nat Genet. 2002;31:106-110.
11. Vogel AM, Weinstein BM. Studying vascular development in the zebrafish. Trends Cardiovasc Med. 2001;10:352-360.
12. Weinstein BM. Blood vessels under construction. Cell. 2002;111:456-458.
13. Weinstein BM. Building the house around the plumbing? Bioessays. 2002;24:397-400.
14. Weinstein BM. Vascular cell biology *in vivo*–a new piscine paradigm? Trends Cell Biol. 2002;12:439-445.
15. Yelon D, Weinstein BM, Fishman MC. Cardiovascular system. In: Solnica-Krezel L, ed. Pattern formation in zebrafish. In: Results and Problems in Cell Differentiation, vol. 40, series editor: Henig, W. New York: Springer, 2002;298-321.

[a]Left during 2001
[b]Left during 2002

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# CONTROL OF GENE EXPRESSION IN PROKARYOTES

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Edit Rutkai, *Guest Researcher*
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Temperate bacteriophages can enter into a long-term and mutually beneficial association with their bacterial hosts. This association is called lysogeny; it is a major mechanism of horizontal gene transfer between bacterial families, and temperate phages constitute a reservoir of genes, such as those encoding virulence factors, that allow their hosts to survive environmental changes. To establish and maintain lysogeny, an infecting phage must carefully control the expression of its own genes so that host viability is unimpaired; at the same time, the phage must synchronize its own replication with that of the host. We report on mechanisms that some phages use to resolve each of these problems.

## Mechanism and Control of Transcription Termination

*King, Sen, Sloan*

The ability of organisms to adjust to environmental stress and to choose between alternative pathways of development requires precise control of gene expression. Transcription, the first step in gene expression, is catalyzed by RNA polymerase (RNAP), a target of many regulators. RNAP is a large, multi-subunit enzyme whose core is structurally and functionally conserved in all kingdoms of life. After the enzyme initiates transcription, it continues to elongate the transcript until it reaches a terminator site. At this point, the enzyme, template, and transcript frequently dissociate from each other such that elongation ceases. Terminators insulate contiguous groups of genes from each other and thus allow independent control of the transcription of each group. In addition, antiterminators can control the efficiency of termination and, hence, the expression of genes downstream of terminators. Termination helps silence virus gene expression in lysogens, and antitermination is used to express these genes during normal virus growth. The molecular mechanisms of both processes remain poorly understood. We are intensively studying an antitermination mechanism found in phage HK022, a virus that parasitizes *E. coli* and uses the host RNAP to express its genes.

We previously showed that phage HK022 uses an antitermination mechanism that differs from any previously described. Each of the left and right transcription units of the HK022 chromosome contains an antiterminator site, *putL* and *putR*, respectively. A nascent *put* transcript binds to the RNAP molecule that catalyzed its synthesis and remains associated with it as the enzyme moves down the template. Association with *put* RNA modifies the enzyme so that it resists termination at downstream terminators. No other protein factor is required for the binding to occur, other RNAP molecules in the same cell are unaffected, and there is no apparent terminator specificity. Antitermination promotes the expression of many genes that are required for virus growth. This particular elongation control pathway appears simpler than others found to date, and its simplicity makes it an attractive target for deeper analysis. We are investigating what is unique about the antiterminator RNA and how it changes the properties of RNAP.

Computer simulations, enzymatic probing, comparison of *putL* to *putR*, and analysis of *put* mutants have all provided clues about the details of *put* RNA structure and their importance for antitermination. Our evidence suggests that the newly synthesized *put* transcript folds into a structure consisting of two stem-loops separated by an unpaired base. Fig. 26 summarizes the results and conclusions of extensive structure-function studies. We have identified a large number of bases and base pairs that are important for antitermination, potentially allowing us to identify new antiterminator sites in other organisms and to understand the role of different regions of the *put* structure.

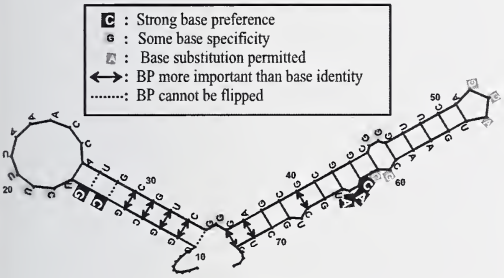
C : Strong base preference
G : Some base specificity
C : Base substitution permitted
⟷: BP more important than base identity
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**FIGURE 26**

Mutational analysis of *put*.

Characterization of RNAP mutants that prevent phage growth by reducing antitermination has provided clues about how the enzyme is modified. Such mutations alter a zinc binding domain (the Zn finger) located near the amino terminus of the β' subunit. The Zn finger contains two pairs of invariant cysteines flanking a moderately well conserved segment of 13 amino acids that is rich in basic residues. We replaced each of the basic residues with alanine and determined the effects of the substitutions on termination, antitermination, and cell viability (Fig. 27). All the mutants were defective in *put*-mediated antitermination, yet the severity of the defect depended on the mutant and the sequence of the upstream stem-loop of put RNA. Some, but not all, mutants distinguished between put variants that differed in this region. Such allele specificity suggests that the Zn finger interacts directly and specifically with *put* RNA in order to anchor the transcript to the translocating enzyme. In fact, the observation that at least one of the mutations (Y75N) prevents association of *put* RNA with RNAP supports this role of the Zn finger. All the mutants with replacements of the basic residues were viable, and those mutant enzymes subjected to testing transcribed and terminated normally *in vitro* on a template that lacked a put site. By contrast, replacement of the invariant cysteines with histidine or alanine prevented cell growth unless a second, functional copy of the gene was also present.

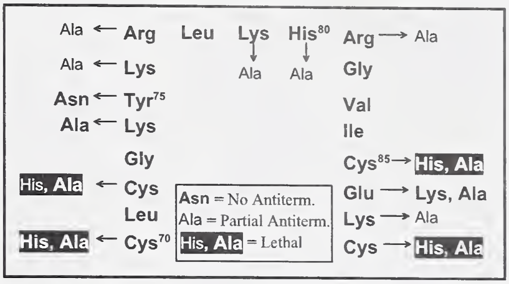

**FIGURE 27**

Phenotypes of Zn finger mutants.

## Evolution of Insertion Site Specificity

*Dorgai, Rutkai*

Many temperate bacteriophages lysogenize their hosts by inserting their chromosomes into specific sites (*attB* or bacterial attachment sites) in the host chromosome, thus ensuring coordinate replication of phage and host chromosomes. Insertion requires integrase, which is a virus-encoded protein that catalyzes recombination between phage and bacterial attachment sites. Bacterial chromosomes contain many *attB*s, each specific to one or a few phages. Although different integrases share a common catalytic mechanism and are related to each other in structure, proteins encoded by different phages typically recombine only their cognate sets of attachment sites. The existing relationships and distribution of integrases and attachment sites imply that phages occasionally change their insertion specificity over an evolutionary time scale. Alteration of insertion specificity cannot occur in a single step because it requires alterations of both integrase and the multiple integrase binding regions within the attachment sites. In addition, efficient insertion requires sequence identity within the "overlap region," a short DNA segment that lies between the points of DNA strand exchange in the attachment sites; in fact, the attachment sites of different phages typically have different overlap regions. We are using two closely related but functionally distinct sets of integrases and attachment sites as a tool to understand how changes in insertion specificity might occur.

We showed that the site specificity of phage λ integrase can be changed to that of phage HK022 by the replacement of five λ residues with their HK022 counterparts. By themselves, two of the replacements relax specificity: the double mutant efficiently recombines both λ and HK022 attachment sites. The other three replacements restrict specificity: the triple mutant recombines neither λ nor HK022 attachment sites but does recombine mutant λ sites that can also be recombined by HK022 integrase. To understand how the mutations alter specificity, we asked if they affected recombination of sites other than those of wild-type λ and HK022. When phage λ infect a cell that lacks *attB*, lysogeny is infrequent, but, when lysogeny does occur, integrase usually catalyzes insertion of the phage chromosome into one of 10 to 20 secondary attachment sites. These sites are equivalent to *attB* mutants. Phage containing either or both of the relaxing *int* mutations lysogenized a host lacking *attB* with about the same frequency as wild type. One of the relaxed mutants differed from and the other was similar to wild type in its preferences for particular secondary sites. We conclude that specificity relaxation by these mutations is sequence-dependent and narrow in scope. The three restricting *int* mutations, which we tested as a triple mutant, strongly reduced the frequency of insertion into all secondary sites; therefore, specificity restriction is broad in scope. Among the few insertion sites that we found, one differed from any used by wild-type phage or the relaxed mutants. Phage that contained all five mutations inserted into secondary sites about twice as frequently as wild type and had new preferences for individual sites. The preferences of the quintuple mutant were not a simple sum of the component mutations, suggesting that interaction among the protein domains affected by the replacements is an important element of specificity alteration.

We used our findings to refine a model for the evolution of phage insertion specificity. The initial step is phage insertion into a secondary attachment site whose overlap region differs from that of the wild-type *attB*. Abnormal excision of this phage from the bacterial chromosome replaces phage DNA with adjacent bacterial DNA and incorporates the new overlap region sequence into the phage attachment site. We propose that this phage variant is adapted to insert efficiently into the secondary host site by a series of relaxing and restricting *int* mutations. According to this model, new attachment sites evolve from sequences that already have some activity as integrase substrates, an attractive feature not found in other models. In addition, the new phage attachment site will be located at one end of the bacterial DNA replacement, where it is usually found in existing phages.

## SELECTED PUBLICATIONS

1. King RA, Madsen PL, Weisberg RA. Constitutive expression of a transcription termination factor by a repressed prophage: promoters for transcribing the phage HK022 *nun* gene. J Bacteriol. 2000;182:456-462.
2. Sen R, King RA, Mzhavia N, Madsen PL, Weisberg RA. Sequence specific interaction of nascent antiterminator RNA with the zinc finger motif of Escherichia coli RNA polymerase. Mol Microbiol.2002;46:215-222.
3. Sen R, King RA, Weisberg RA. Modification of the properties of elongating RNA polymerase by persistent association with nascent antiterminator RNA. Mol Cell. 2001;7:993-1001.

[a] Arrived during 2002

[b] Left during 2002

# CHROMATIN-BASED EPIGENETIC STRUCTURES IN THE CONTROL OF CELL DIFFERENTIATION AND PROLIFERATION

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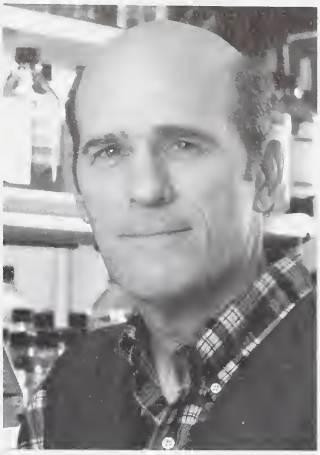

Our research focuses on the role of higher-order chromatin structure in regulating gene expression. A topic of special interest is epigenetic memory, both how it is established and the determinants of its stability. The central hypothesis to be tested is that defects in epigenetic memory contribute to aging, cancer, and age-related diseases. To test that hypothesis, we are pursuing two areas of inquiry. First, we are developing techniques to assess the type and extent of chromatin remodeling that accompanies developmental programs, terminal differentiation, or senescence. Second, we perform structure-function studies to understand the roles that specific chromatin regulatory gene products may play in the establishment or maintenance of epigenetic memory.

### Genome Sampling to Map Higher-Order Chromatin Domains and Regions of Chromatin Remodeling

*Russanova, Howard*

Several examples in the literature provide direct or indirect evidence for chromatin remodeling in conjunction with differentiation, cancer, or aging. Most such examples derive from serendipitous observations; thus, we have only a limited understanding as to the extent and types of chromatin changes that may occur in these contexts. Differential display is well established as a systematic means to screen for alterations in mRNA levels. We have combined chromatin immunoprecipitation (ChIP) with a modified form of differential display in order to screen for regions of unusual chromatin structure or domains involved in chromatin remodeling. To date, most of our ChIP experiments have involved chromatin fractionation according to histone acetylation level. Elevated levels of acetylation are typically associated with active gene transcription, whereas underacetylation correlates with repression and silencing. The human genome is organized into alternating gene-rich and gene-poor regions. The observation that markedly underacetylated loci identified in the screens reside uniformly within gene-poor regions is an important validation of the ChIP genome sampling approach. By contrast, highly acetylated loci lie predominantly within gene-rich regions. The differences are highly significant and would not be observed if screening yielded loci at random with respect to higher-order chromatin domains.

The HL-60 promyelocytic cell line has served as a useful model system in which to search for domains of chromatin remodeling. HL-60 cells differentiate into macrophage-like cells in response to treatment with phorbol esters. A screen of several thousand loci revealed several in which terminal differentiation along the macrophage lineage is accompanied by elevated histone acetylation. One such locus, located on chromosome 2 at a position 40 kilobases upstream from the EML4 gene locus, exhibits an average six-fold increase in acetylation. The success of genome sampling in the HL-60 system provides a solid basis for proceeding to searches for chromatin remodeling in the contexts of development, cancer, and aging.

### Histone H3 Methylation Marks the Murine H19-Igf2–Imprinted Region

*Tchernov; in collaboration with Stewart, Pfeifer*

The murine H19-Igf2 imprinted region is characterized by maternal expression of the H19 locus versus paternal expression of the Igf2 gene. A differentially methylated region (DMR) is located upstream from the H19 locus. In maternal cells, the DMR is unmethylated, binds to the transcription factor CTCF, and functions as an insulator to prevent activation of the Igf2 promoter by enhancers located downstream from H19. Conversely, in paternal cells, the DMR is methylated and repressed with respect to transcriptional and insulator activities. Recent evidence indicates that histone H3 methylation at lysine position 9 (K9) can precede DNA methylation as a mark for transcriptional silencing. Such is the case in diverse systems ranging from DNA methylation in *Neurospora* and *Arabidopsis* to X-chromosome silencing in mammalian embryonic stem cells. Experiments by members of our group and collaborators revealed a high level of histone H3-K9 methylation near the H19-associated DMR and promoter region in paternal but not maternal cells. By contrast, we detected H3-K9 methylation at control regions for the Igf2 locus in paternal cells only. Histone H3 methylation at lysine position 4 (K4) is typically associated with transcriptional activation. We found highly localized H3-K4 methylation in the Igf2 promoter in paternal cells, but not in maternal cells. Each of these results is consistent with a role of histone H3 K4/K9 methylation in control of imprinted genes. Of further importance is the fact that the characterization of selective histone methylation at the Igf2 locus provides a reference, or positive control, for genome sampling to detect changes in H3 methylation in the contexts of development and age-related processes.

### Association of Hox-a10 and Sirtuin2

*Bae*

As noted above, histone deacetylation is typically associated with transcriptional silencing. Numerous transcriptional repressors recruit RPD3-family deacetylases. Previous work by our group and others revealed that members of this family, including HDAC1, HDAC2, and HDAC3, function within multiprotein complexes to mediate repression. The role of RPD3 deacetylases in epigenetic silencing, as opposed to transcriptional repression, appears to be complex. In some cases, inhibitors of RPD3 deacetylases such as sodium butyrate and trichostatin A can disrupt silencing. On the other hand, deletion of RPD3 and RPD3-like deacetylases in budding yeast enhances rather than impairs silencing. The NAD-dependent deacetylase SIR2 is essential for silencing in budding yeast, and SIR2-like proteins constitute a highly conserved family ranging from yeast to mammals. To date, however, no evidence has been reported that Sirtuins, the mammalian SIR2 homologs, function in transcriptional regulation. Experiments performed by our group, using the yeast two-hybrid system, co-immunoprecipitation of *in vitro* translated products, and co-immunoprecipitation from mammalian cell extracts, revealed that Sirtuin2 interacts with the homeodomain protein Hox-a10. Further, over-expression of Sirtuin2 counteracts transcriptional activation by Hox-a10. These results establish a link between mammalian SIR2 homologs and transcriptional control. In addition, given that Hox-a10 plays a role in genito-urinary tract formation, the results suggest the possibility that Sirtuin2 may modulate the establishment or maintenance of repressive chromatin domains during development.

## SELECTED PUBLICATIONS

1. Howard B. Chromatin, aging, and cell senescence. In: Hisama F, Weissman S, Martin G, eds. Chromosomal instability and aging;in press.

2. Humphrey GW, Wang Y, Russanova VR, Hirai T, Qin J, Nakatani Y, Howard BH. Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. J Biol Chem. 2001;276:6817-6824.

## COLLABORATORS

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# DEVELOPMENTAL GENE REGULATION OF THE IMMUNE SYSTEM

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We are interested in transcription factors that regulate the development of the immune system. We previously isolated ICSBP/IRF-8, a DNA-specific transcription factor expressed in hematopoietic cells, and showed that it regulates the development of bone marrow progenitor cells to generate macrophages and granulocytes. We also showed that the introduction of ICSBP rescues normal growth and differentiation of these cells in ICSBP-null progenitors. Our studies have investigated the effect of ICSBP on the development and maturation of dendritic cells (DCs). We have also identified target genes regulated by ICSBP and characterized ICSBP's role in controlling target promoters and regulating the activity of chromatin.

We have studied the role of chromatin from another perspective and characterized a bromodomain protein Brd4. Brd4 is a novel member of the conserved BET family and contains a motif indicative of interaction with chromatin. We showed that, consistent with a strong affinity for chromatin, it associates with mitotic chromosomes. Recent FRET and FLIP analyses suggest that Brd4 and a related protein interact with acetylated histones in living cells. Conditional knock-out and antisense/RNAi approaches further elucidate the role of Brd4, particularly in cell growth.

### Microarray Identification of Genes Controlled by ICSBP during Macrophage Differentiation and Role of Chromatin

*Tamura, Laricchia, Thotokura; in collaboration with Ko*

ICSBP-/- mice develop a syndrome similar to human chronic myelogenous leukemia (CML). In addition, ICSBP-/- macrophages are functionally defective, causing the mice to be immunodeficient. By studying bipotential ICSBP-/- myeloid progenitor cell lines, we found that ICSBP drives their differentiation toward mature functional macrophages while causing a complete growth arrest. On the other hand, it inhibits granulocytic differentiation, explaining the CML-like syndrome in ICSBP-/- mice.

To study the molecular mechanism by which ICSBP controls myeloid cell differentiation, we performed a genome-wide gene expression analysis by using the mouse 15K cDNA microarray. To analyze a change in gene expression patterns at an early stage, we used an inducible ICSBP/estrogen receptor (ICSBP/ER) chimera. We identified 27 known genes and 28 unknown genes that displayed a greater than two-fold increase or decrease within four hours of ICSBP expression. Among them, three genes including cystatin C were induced early, even in the presence of cycloheximide, indicating that they are direct targets of ICSBP. By using a newly developed self-inactivating retrovirus-based reporter assay, we observed that ICSBP/ER induces promoter activation. ICSBP/ER down-regulated the expression of the *c-myc* gene, but cycloheximide inhibited the repression, indicating that ICSBP regulates *c-myc* gene expression through an ICSBP-inducible molecule or molecules. We found that the transcription factor Blimp-1, which is implicated in macrophage differentiation, growth inhibition, and *c-myc* repression, was directly induced by ICSBP, suggesting that ICSBP down-regulates *c-myc* through Blimp-1. The results indicate that ICSBP controls the expression of regulatory genes as well as those involved in macrophage function. Microarray analysis is thus a useful first step toward understanding global changes in gene expression patterns during differentiation of immune cells.

Introduction of ICSBP into progenitor cells causes dramatic alterations in the nuclear architecture, suggesting that ICSBP interacts with chromatin to alter target gene expression. Interaction of ICSBP with chromatin during macrophage differentiation is under study with fluorescent recovery after photobleaching.

### Role of ICSBP in the Development of Dendritic Cells

*Tsujimura, Tamura, Kong*

Dendritic cells (DCs) develop from bone marrow (BM) progenitor cells and mature in response to external signals to elicit functions important for innate and adaptive immunity. We found that ICSBP-/- mice lack subsets of DCs (pDCs and CD8α$^+$ Dcs). To investigate the role of ICSBP in DC development, we employed a Flt3 ligand-based *in vitro* culture system. DCs that developed *in vitro* from *ICSBP-/-* BM cells were defective in the expression of MHC class II and co-stimulatory molecules, indicating that differentiation of *ICSBP-/-* progenitors toward immature DCs is impaired. Moreover, -/- DCs displayed a generalized maturation failure and did not express IL-12 p40 mRNA and proteins in response to various maturation signals, consistent with the feature observed with *ICSBP-/-* DCs *in vivo*. In addition, -/- DCs failed to increase expression of MHC class II and co-stimulatory molecules upon maturation signals. We show that retroviral introduction of ICSBP restores the ability of -/- progenitors to develop immature DCs that can undergo full maturation upon activation signals. Transduction of the wild-type ICSBP, but not transcriptionally inactive mutants, increased expression of MHC class II and co-stimulatory molecules on immature cells. More important, ICSBP-transduced cells produced IL-12 proteins upon activation, along with increased expression of co-stimulatory molecules. The study identifies ICSBP as a factor critical for both early differentiation and final maturation of DCs.

### Interaction of Bromodomain Protein Brd4 with the DNA Replication Machinery and the Role in the G1- S Cell Cycle Progression

*Farina, Dey, Jang; in collaboration with Hurwitz*

Brd4 belongs to the BET family of nuclear proteins that carry two bromodomains thought to be implicated in interactions with chromatin. Expression of Brd4 correlates with cell growth and is induced during early G1 phase upon mitogenic stimuli. To study the role of Brd4 in cell growth control, we examined the effects of over-expression of Brd4 in NIH-3T3 and HeLa cells. We found that ectopic Brd4 expression inhibits cell cycle progression from G1 to S. Co-immunoprecipitation experiments showed that endogenous and transfected Brd4 interact with the replication factor C (RFC), the conserved five-subunit complex essential for DNA replication. *In vitro* analysis demonstrated that Brd4 directly binds to the largest subunit, RFC-140, thereby interacting with the entire RFC. In line with the inhibitory activity seen *in vivo*, recombinant Brd4 inhibited RFC-dependent DNA elongation reactions *in vitro*. Analysis of Brd4 deletions indicated that both the interaction

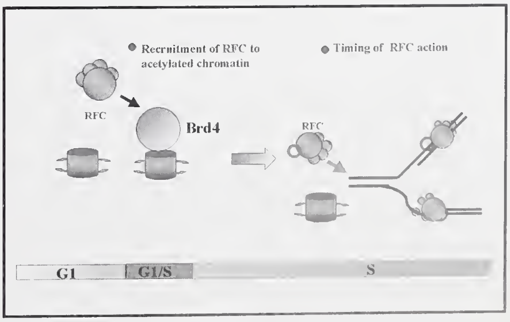

FIGURE 28

Model of Brd4 action

with RFC-140 and the inhibition of entry into S phase depend on the second bromodomain of Brd4. Finally, supporting the functional importance of this interaction, co-transfection of RFC-140 reduced the growth-inhibitory effect of Brd4. Taken together, the present study suggests that Brd4 regulates cell cycle progression in part by interacting with RFC (Fig. 28). A systematic biochemical analysis is under way to identify proteins with which Brd4 forms a complex.

### Interaction of Brd4 with Acetylated Chromatin

*Kanno T, Kanno Y, Dey; in collaboration with Lenardo, Siegel*

To assess the interaction of Brd4 with chromatin, we have carried out analysis of fluorescent loss in photobleaching (FLIP) of transiently transfected GFP-Brd4 in the presence or absence of TSA, a histone deacetylase inhibitor. We found that TSA treatment inhibits loss of fluorescence, indicating that Brd4 preferentially interacts with chromatin. Biochemical analysis with Brd4-nucleosome interactions also supports strong affinity of Brd4 for acetylated histones. Interaction of the BET family proteins with chromatin is also under investigation with the fluorescence resonance energy transfer technique (FRET).

### Dynamic Relationship between Histone Acetylases and Deacetylases

*Kanno T, Kanno Y; in collaboration with Howard, Lenardo, Siegel*

Histone acetylases (HAT) and deacetylases have opposing enzymatic activities. Both enzymes affect chromatin structures and regulate transcription. It is assumed that a mechanism balances the activities of the two enzymes in the cell. We hypothesized that one way to achieve balance is for the two enzymes to interact with each other and function as a single entity. By FRET analysis, we showed that the acetylase PCAF and deacetylase HDAC1 are spatially proximate in living cells compatible with their physical interaction. In agreement, co-immunoprecipitation assays demonstrated that endogenous HDACs are associated with PCAF and another acetylase GCN5 in HeLa cells. We found by glycerol gradient sedimentation analysis that HATs are integrated into a large multi-protein HDAC complex that is distinct from the previously described HDAC complexes containing mSin3A, Mi-2/NRD, or CoREST. The HDAC-HAT association is partly accounted for by a direct protein-protein interaction observed *in vitro*. The HDAC-HAT complex may play a role in establishing a dynamic equilibrium of the two enzymes *in vivo*.

## SELECTED PUBLICATIONS

1. Aliberti J, Shulz O, Pennington DJ, Tusjimura H, Ozato K, Sher A. Essential role for ICSBP in the in vivo development of murin CD8$\alpha$+ dendritic cells. Blood. 2002;(e-publication ahead of print).
2. Kuwata T, Gongora C, Kanno Y, Sakagichi K, Tamura T, Basrur V, Martinez R, Appella E, Golub T, Ozato K. Gamma interferon triggers interaction between ICSBP (IRF-8) and TEL, recruiting the histone deacetylase HDAC3 to the interferon responsive element. Mol Cell Biol. 2002;22:7439-7448.
3. Lefebvre B, Brand C, Lefebvre P, Ozato K. Chromosomal integration of retinoic acid responsive element prevents cooperative activation of RAR and RXR. Mol Cell Biol. 2002;22:1446-1459.
4. Lefebvre B, Ozato K, Lefebvre P. Phosphorylation of histone H3 is functionally linked to retinoic acid receptor $\beta$ promoter activation. EMBO Rep. 2002;3:335-340.
5. Maruyama T, Dey A, Farina A, Cheong J, Bermudez VP, Tamura T, Sciortino S, Shuman J, Hurwitz J, Ozato K. A mammalian bromodomain protein Brd4 interacts with the replication factor C and inhibits progression to S phase. Mol Cell Biol. 2002;22:6509-6520.
6. Masumi A, Ozato K. Coactivator p300 acetylates the interferon regulatory factor-2 in U937 cells following phorbol ester treatment. J Biol Chem. 2001;276:20973-20980.
7. Tamura T, Ozato K. ICSBP/IRF-8: its regulatory roles in the development of myeloid cells. J Interferon Cytokine Res. 2002;22:145-152.

8. Tusjimura H, Nagamura-Inoue T, Tamura T, Ozato K. IFN consensus sequence binding protein/IFN regulatory factor-8 guides bone marrow progenitor cells toward the macrophage lineage. J Immunol. 2002;169:1261-1269.
9. Tsujimura H, Tamura T, Gongora C, Aliberti J, Reis e Sousa C, Sher A, Ozato K. ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development. Blood. 2002;(e-publication ahead of print).

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# REGULATION OF DNA REPLICATION AND GENE EXPRESSION DURING ANIMAL DEVELOPMENT

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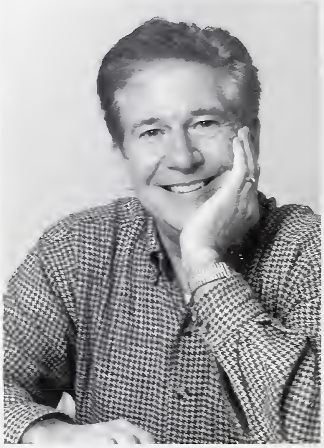

We have focused on those aspects of eukaryotic DNA replication that appear to be unique to the metazoa: the mechanism by which metazoa regulate the number and locations of initiation sites during cell proliferation and development.

## Eukaryotic DNA Replication

*DePamphilis, Li, Kong, Bogan, Sun, Zhang; in collaboration with Coleman*

In previous studies, we were the first to map origins of bi-directional replication (OBRs) to specific chromosomal sites of 0.4 to 2 kb in mammalian cells, and we demonstrated that pre-replication complexes are assembled at or close to these sites when hamster cells transit from metaphase to G1 phase during cell division. Moreover, we discovered that one of the six subunits (Orc1) of the "origin recognition complex" (ORC) that is responsible for determining where replication begins is selectively released from chromatin before or during metaphase and then rebinds to chromatin during the M to G1 transition, resulting in the appearance of pre-replication complexes at specific genomic sites. This discovery suggested that ORC determines not only where but also when replication begins by preventing assembly of pre-replication complexes until mitosis is complete and a nuclear membrane has reformed. Such an "ORC cycle" would represent a novel mechanism for regulating cell division and the premier step in regulating the onset of DNA replication.

During the past year, we have addressed two critical questions: whether ORC recognizes specific, genetically required sequences in complex eukaryotic replication origins, and whether ORC subunits cycle on and off chromatin during the cell division cycle.

Fission yeast (*S. pombe*), like mammals, contains large, AT-rich replication origins that lack a recognizable consensus sequence, but that nevertheless bear sequences required for replication. We have discovered that the SpOrc4 subunit was solely responsible for selection of initiation sites in *S. pombe*. Others had shown that Orc4 contains a special AT-hook motif that binds to AT-rich DNA sequences. We found that *S. pombe* ORC binds to specific sites within *S. pombe* replication origins that are genetically required for origin activity and that site selection is determined solely by the Orc4p subunit. Consistent with the fact that Orc4 contains nine AT-hook motifs that others have shown to bind to AT-rich sequences, these sites consist of clusters of A or T residues on one strand but are devoid of either alternating A and T residues or GC-rich sequences. We have further shown that Orc4p binds specifically to only one of the four required sequences in ARS3001, where it initiates assembly of a pre–RC and initiates bi-directional DNA replication. Thus, *S. pombe* replication origins may provide an appropriate paradigm for replication origins in higher eukaryotes.

Binding of ORC to DNA (chromatin) is the first step in assembly of a pre-replication complex. In contrast to yeast, in which all six ORC subunits are stably bound to chromatin throughout the cell cycle, mammalian Orc1 is selectively released from chromatin during S phase. Moreover, the Orc1 that is released during S phase is rapidly ubiquitinated and in some cases degraded. Other ORC subunits remain stably bound to chromatin and are not substrates for ubiquitination. During the M to G1 transition, Orc1 rebinds tightly to hamster ORC/chromatin sites to allow assembly of pre-replication complexes. The sites are located at specific genomic loci referred to as "origins of bi-directional replication." The role of ubiquitination is to sequester Orc1 during S phase and thus prevent reinitiation at replication origins during a single cell division cycle, also providing a mechanism for reprogramming replication origins during animal development or after DNA damage, in which the Orc1 subunit could be degraded. Thus, in contrast to yeast, mammals use a novel pathway to regulate initiation of DNA replication: ORC activity is regulated during each cell division cycle through selective dissociation and reassociation of Orc1 from chromatin-bound ORC.

In searching for the trigger that releases Orc1 from mammalian chromatin, we discovered that the entire *Xenopus* ORC rapidly binds to the somatic cell chromatin, initiates DNA replication, and is released as soon as Mcm proteins are bound to chromatin to form a pre-replication complex.

### Gene Expression at the Beginning of Mammalian Development

*DePamphilis, Vassilev, Kaneko, Zhang; in collaboration with Zhao*

In an effort to identify specific *cis*-acting sequences and *trans*-acting factors that regulate DNA replication and gene expression at the beginning of mouse development, we previously microinjected plasmid DNA into the nuclei of oocytes, fertilized eggs, and two-cell embryos and then measured the ability of the cells either to replicate the DNA or express a reporter gene. The work led to the discovery of a novel transcription factor, mTEAD-2, that is expressed at the onset of zygotic gene expression (ZGE) where it is capable of strongly stimulating transcription from promoters or enhancers that contain its sequence-specific binding site. mTEAD-2 is the only member of the TEAD family of transcription factors that is expressed in mouse embryos during the first seven days of development. Our current goals are to identify the factors that regulate mTEAD-2 gene expression, to elucidate mechanisms by which mTEAD-2 regulates gene expression, and to identify the role of mTEAD-2 in mammalian development.

Investigation of the regulatory region of *mTEAD-2* led to the surprising discovery of another gene only 3.8 kb upstream of *mTEAD-2*. The new gene is a single-copy, testis-specific gene called *Soggy* (*mSgy*) that is transcribed in the direction opposite to *mTEAD-2*, thus placing the regulatory elements of the two genes close to one another. *mSgy* contains three methionine codons that have the potential to act as translation start sites, but most mSGY protein synthesis *in vitro* was initiated from the first Met codon to produce a full-length protein, suggesting that mSGY normally consists of 230 amino acids (26.7 kDa). Transcription begins at a cluster of nucleotides about 150 bp upstream of the first Met codon by involving a TATA-less promoter contained within the first 0.9 kb upstream to produce a single, dominant mRNA of about 1.3 kb. The activity of the promoter is repressed by upstream sequences between -0.9 and -2.5 kb in cells that do not express *mSgy*, but the repression is relieved in cells that do express *mSg*. *mSgy* mRNA is detected in embryos only after day 15 and in adult tissues only in the developing spermatocytes of seminiferous tubules, suggesting that *mSgy* is a spermatocyte-specific gene. Given that *mTEAD-2* and *mSgy* are not expressed in the same cells, the *mSgy/mTEAD-2* locus provides a unique paradigm for differential regulation of gene expression during mammalian development.

We have recently identified the long sought-after co-activator of the TEAD family of transcription factors. TEAD-2/TEF-4 protein purified from mouse cells was associated predominantly with a novel TEAD-binding domain at the N-terminus of YAP65, a powerful transcriptional co-activator. YAP65 interacted specifically with the C-terminus of all four TEAD proteins. Both this interaction and sequence-specific DNA binding by TEAD were required for transcriptional activation in mouse cells. Expression of YAP in lymphocytic cells that normally do not support TEAD-dependent transcription (e.g., MPC11) resulted in up to 300-fold induction of TEAD activity. Conversely, TEAD over-expression "squelched" YAP activity. Therefore, the C-terminal acidic activation domain in YAP is the transcriptional activation domain for TEAD transcription factors. However, while TEAD was concentrated in the nucleus, excess YAP65 accumulated in the cytoplasm as a complex with the cytoplasmic localization protein, 14-3-3. Given that TEAD-dependent transcription was limited by YAP65 and YAP65 also binds Src/Yes protein tyrosine kinases, we propose that YAP65 regulates TEAD-dependent transcription in response to mitogenic signals.

## SELECTED PUBLICATIONS

1. DePamphilis ML. DNA replication in humans. In: Kazazian HH, Creighton TE, eds. Wiley encyclopedia of molecular medicine. New York: John Wiley and Sons, 2002;1080-1086.
2. DePamphilis ML. Eukaryotic DNA replication forks. In: DePamphilis M, ed. Eukaryotic DNA replication. Baltimore: ChemTracts–Biochemistry & Molecular Biology, Data Trace Publishing, 2002;313-325. 3.
3. DePamphilis ML. Eukaryotic replication origins and initiation of DNA replication. In: Encyclopedia of life sciences. London: Macmillan Reference Ltd., 2001;www.els.net.
4. DePamphilis ML, Kaneko KJ, Vassilev A. Activation of zygotic gene expression in mammals. Adv Dev Biol Biochem. 2002;12:55-84.
5. Kong D, DePamphilis ML. Site-specific DNA binding of the *Schizosaccharomyces pombe* origin recognition complex is determined by the Orc4 Subunit. Mol Cell Biol. 2001;21:8095-8103.
6. Kong D, DePamphilis ML. Site-specific ORC binding, pre-replication complex assembly, and DNA synthesis at *S. pombe* replication origins. EMBO J. 2002;21:5567-5576.
7. Li CJ, DePamphilis ML. Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S to M transition in the cell division cycle. Mol Cell Biol. 2002;22:105-116.
8. Sun WH, Coleman TR, DePamphilis ML. Cell cycle dependent regulation of the association between origin recognition proteins and somatic cell chromatin. EMBO J. 2002;21:1437-1446.
9. Sun WH, DePamphilis ML. Methods for detecting cells in S-phase. In: Lieberman H, ed. Cell cycle checkpoint control protocols. Totowa, NJ: Humana Press, 2002;in press.
10. Vassilev A, Kaneko KJ, Shu H, Zhao Y, DePamphilis ML. TEAD(TEF) transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. Genes Dev. 2001;15:1229-1241.

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# MECHANISMS OF EUKARYOTIC RNA EXPRESSION AND FUNCTIONS OF THE HUMAN La ANTIGEN

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We study mechanisms of eukaryotic RNA biogenesis and functions of the human La antigen, for which transcription by RNA polymerase III (Pol III) serves as a model system of gene expression. Transfer RNAs are the Pol III transcripts currently under most intense study. The precursors of tRNAs are synthesized as nascent transcripts that undergo a "maturation" process involving a series of coordinated enzymatic and intracellular trafficking activities that ultimately deliver a functional tRNA to the ribosomes. Our interests focus on a few of the individual steps in RNA production (e.g., transcription initiation and termination as well as RNA processing and intranuclear trafficking), including coordination of the sequential steps in time and space within the cell. In particular, we have focused on the human La antigen, a protein that is a target of autoantibodies in patients suffering from certain autoimmune disorders (e.g., systemic lupus erythematosus). The human La antigen is a nuclear-cytoplasmic shuttling protein that also binds to the 5' untranslated regions of certain mRNAs, regulating and coordinating key steps in RNA expression. We use modern genetics, molecular biology, and biochemical approaches and rely heavily on analytical biochemistry as well as tissue culture, yeast systems, and transgenic and gene-altered mice.

### Functions of the Human La Antigen in RNA Expression

*Maraia, Intine, Schwartz, Mukhergee, Muse, Nelson, Treanor*

Human La antigen is a nuclear phosphoprotein that is present in all eukaryotic cells thus far examined. As alluded to above, La protein is a target "antigen" of antibodies (Ab) in patients suffering from autoimmune disorders such as systemic lupus erythematosous (SLE, Lupus), neonatal lupus, and Sjögren's syndrome. Evidence from our and other laboratories indicates that La is a component of a Pol III holoenzyme, which remains associated with newly synthesized transcripts to direct their maturation. As such, La is considered a regulatory chaperone for nascent RNAs because it can control their nuclear residence and accessibility to the processing enzymes. In part, La functions as a regulatory chaperone by sequence-specific binding to a UUU-OH 3' terminal motif that is common to all transcripts synthesized by Pol III and that results from transcription termination by RNA Pol III.

We mapped the major phosphorylation site of La to serine 366 (S366) and showed that the phosphorylation interferes with La's ability to interact with the initiating pppG of the nascent transcript and to activate transcription initiation by Pol III, implying that these activities are mechanistically related. The results suggest an

"La cycle" of transcription and post-transcriptional regulation that is mediated in part by the C-terminal domain (CTD) of La. Others have recently shown that La is dephosphorylated at S366 during early apoptosis. We have developed two sets of monospecific antibodies that can distinguish phosphoS366 (pLa) from nonphospohoS366 (npLa). We could demonstrate that npLa and pLa exhibit distinct subnuclear localizations (nucleoplasm versus nucleoli) and that they are differentially associated with certain RNAs *in vivo*.

Our data point to several trafficking signals in La that control nuclear, nucleolar, and cytoplasmic localization. Using a tRNA suppressor reporter system, we have identified an intranuclear trafficking defect that is associated with detrimental disordering of tRNA processing activities. In this case, La recognizes and normally binds to its substrate pre-tRNAs, but the defect causes improper routing in the nucleus, resulting in the accumulation of a dead-end tRNA processing intermediate that is nonfunctional. Our studies revealed that La traffics through the nucleus and is exported to the cytoplasm, and have uncovered an unexpected nuclear export signal (NES) in La. The existence of an efficient, carrier-mediated NES system for La is surprising because the established functions of La in Pol III transcript biogenesis are entirely intranuclear. Further characterization revealed that both the nuclear export pathway used for La and other features of La's NES function are characteristic of some RNA-binding proteins that carry certain mRNAs to the cytoplasm. Accordingly, we have begun to extend our studies to include examination of the involvement of La in the expression of certain target mRNAs, especially those whose expression is critical to growth and development.

### Transcription Termination by RNA Polymerase III

*Maraia, Huang, McGillicuddy, Mozlin*

As indicated above, La protein binds to the 3' terminal motif of nascent transcripts, UUU-OH, that results from termination by RNA Pol III. We have developed a Pol III transcription system in the fission yeast *S. pombe*. A pol III-dependent gene encodes an opal suppressor tRNA that suppresses a nonsense codon in the mRNA encoding a purine-synthetic enzyme (*Ade6-704*); the enzyme's activity can be monitored by using an *in vivo* colorimetric plate assay. We demonstrated that the expression of the gene in *S. pombe* depends on accurate and efficient termination by pol III and established that the minimal number of dT residues required for efficient termination is five. We have uncovered an intriguing correlation between the sensitivity of RNA Pol III to the toxin $\alpha$-amanitin and the sensitivity to the Pol III termination signal and have begun a structure-function analysis of the largest subunit of Pol III by using our *in vivo* reporter system.

We have shown that the tRNA gene under study requires the La protein for efficient expression *in vivo*. Many important questions remain, such as the mechanistic link between La and pol III termination; whether the lack of reporter gene–derived transcripts in the La-minus strain is attributable to a defect in transcription rate, nascent RNA processing, or both; and the nature of other factors that contribute to the La-dependent activation of this tRNA gene.

### Transcription Initiation by RNA Polymerase III

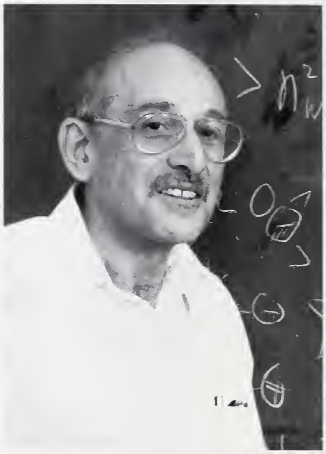
*Maraia, Huang, Mozlin*

Though long interested in transcriptional termination, we have made some recent discoveries that have led us to examine mechanisms of Pol III initiation. Specifically, ongoing studies focus on the two central transcription factors (TF), TATA-binding protein (TBP) and the TFIIB-related factor, Brf, which both function at the core promoter. Homologs of these factors exist in all eukaryotes and archaea and play a central role in controlling transcription initiation. The core promoter is itself of central importance in transcription because, in addition to directing transcription initiation, core promoters integrate complex input signals from distal regulatory elements. Genome-wide analysis has revealed that, in contrast to the core promoters of human and *S. cerevisiae* tRNA genes, which have long been known to be TATA-less, the core promoters of tRNA and 5S rRNA genes in *S. pombe* contain TATA elements. We used tRNA-dependent suppression and other *in vivo* assays as well as *in vitro* transcription to demonstrate an obligatory requirement for upstream TATA elements for tRNA and 5S rRNA expression in *S. pombe*. We extended the results to large rRNA synthesis, as mutation of the TATA element in the Pol I promoter also abolished rRNA expression in fission yeast. The results point to TATA-unified transcription systems in contemporary eukaryotes and provide insight into the residual need for TBP by all three RNA polymerases in other eukaryotes despite a lack of TATA elements in their promoters.

## SELECTED PUBLICATIONS

1. Hamada M, Huang Y, Lowe TM, Maraia RJ. Widespread use of TATA elements in the core promoters for RNA polymerases III, II, and I in fission yeast. Mol Cell Biol. 2001;21:6870-6881.
2. Hamada M, Sakulich AL, Koduru SB, Maraia R. Transcription termination by RNA polymerase III in fission yeast: a genetic and biochemically-tractable model system. J Biol Chem. 2000;275:29076-29081.
3. Huang Y, Hamada M, Maraia RJ. Isolation and cloning of four subunits of a fission yeast TFIIIC complex that includes an ortholog of the human regulatory protein TFIIICbeta. J Biol Chem. 2000;275:31480-31487.
4. Huang Y, Hamada M, Patel J, Maraia RJ. Construction of FLAG and histidine tagging vectors for Schizosaccharomyces pombe. Yeast. 2001;18:463-468.
5. Huang Y, Maraia RJ. Comparison of the RNA polymerase III transcription machinery in *S. pombe*, *S. cerevisiae* and humans (review). Nucl Acids Res. 2001;29:2675-2690.
6. Intine RV, Dundr M, Misteli T, Maraia RJ. Aberrant nuclear trafficking of La protein leads to disordered processing of associated precursor tRNAs. Mol Cell. 2002;9:1113-1123.
7. Intine RVA, Sakulich AL, Koduru SB, Huang Y, Pierstorrf E, Goodier JL, Phan L, Maraia RJ. Transfer RNA maturation is controlled by phosphorylation of the human La antigen on serine 366. Mol Cell. 2001;6:339-348.
8. Maraia R. The human La antigen [SS/B]. In: Creighton TE, ed. Wiley encyclopedia of molecular medicine. New York: John Wiley & Sons, 2002;1883-1886.
9. Maraia RJ. La protein and the trafficking of nascent RNA polymerase III transcripts. J Cell Biol. 2001;153:F13-F17.
10. Maraia RJ, Intine RV. La protein and its associated small nuclear and nucleolar precursor RNAs. Gene Expr. 2002;10:41-47.
11. Maraia RJ, Intine RV. Recognition of nascent RNA by the human La antigen: conserved and diverged features of structure and function. Mol Cell Biol. 2001;21:367-379.

# PHYSICAL FORCES ORGANIZING BIOMOLECULES



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Thomas Zemb, Ph.D., *Guest Researcher*

Our research efforts focus on polymer-coated lipids used in drug delivery, DNA/lipid assemblies for gene therapy, DNA assemblies such as are seen in viral capsids and *in vitro,* polypeptides and polysaccharides in suspension, and lipid/water liquid-crystals. In all these systems, we simultaneously observe the structure of packing and measure intermolecular forces or interaction energies. We would like to characterize and codify measured forces in order to build a practical molecular physics of living systems. Our undertaking is strengthened by its strong connection with physical theory, in particular statistical mechanics applied to liquid crystals and complex fluids.

Acting in liquids, measured forces depend on temperature, salts, solutes, and specific ligands; we speak of the forces as "thermodynamic" forces, whose strengths and specificities are exquisitely controlled by cellular and solutions conditions. Their changes with water chemical potential reveal hydration; their dependence on solute chemical potentials indicate "excess" or "deficit" due to solute attraction or repulsion. We can think of them as "direct," e.g., electrostatic, van der Waals, or hydration forces, or as "indirect,", acting through changes in the translation and configuration free energy of concentrated macromolecules.

### Van der Waals Forces: Ions, Lipids, and Membrane Interactions

*Podgornik, Hansen, Petrache, Zemb, Dubois; in collaboration with French, Nagle*

Normally in the domain of physical field theory, primarily recognized in chemical engineering, van der Waals or "charge-fluctuation" forces act in striking ways among biological materials, ways that are often, however, disregarded. As the dominant force between hydrocarbons. they "cohere" the molecules that compose membranes and contribute to the powerful surface tension at membrane interfaces. At the next level, interactions between membranes, van der Waals forces are the dominant, perhaps sole, attraction that creates membrane multilayers or allows membranes to adhere to artificial surfaces. Similarly, in proteins, van der Waals forces are locally weak attractions that, in their ubiquity, sum to cohere and stabilize protein.

Because of the difficulty of the underlying physical theory, van der Waals forces have not, for the most part, been treated rigorously. Moreover, it has been difficult to design measurements that test their strength and consequence. Our work during the past year has extended to the theoretical physics of more general mathematical equations to compute van der Waals interactions as well as to the "wet chemistry" of making preparations under which these forces can be systematically observed. One unexpected bonus has been the initiation of a collaboration with engineers that takes advantage of our equations to design production procedures for thin-film resistors in computer chips. We expect the collaboration to yield experimental data to be used in computing van der Waals forces.

People usually think of ions as particles whose primary properties are determined by their electric charge. In fact, given that ions vary widely in their effects on biological materials, ion "specificity" beyond simple charge properties is a major issue in biology. One overlooked property of ions is polarizability, the ability of the charge to shift or fluctuate, a property seen in charge fluctuation forces. During the past year, we have been using ion polarizability to explain the difference between chloride, bromide, iodide, nitrate, and other negative ions that seem to bind to membranes. Our collaborators in France have seen that positively charged membranes often collapse in the presence of the larger ions that adhere to them. Normally, ions do not go near the poor solvent interiors of membranes. Van der Waals forces that are proportional to ion size seem to be the reason for the extra attraction. At the NIH, we have begun to observe how the attraction between membranes varies when salts of different ions, i.e., chloride versus bromide, are dissolved in the intervening water. Membrane multilayers swell by 50 percent with bromide but not with chloride. As already pointed out, at least one important factor in ion discrimination is the neglected van der Waals force. From such membrane measurements, we are following a new strategy to understand puzzling properties of ions with many types of biological materials.

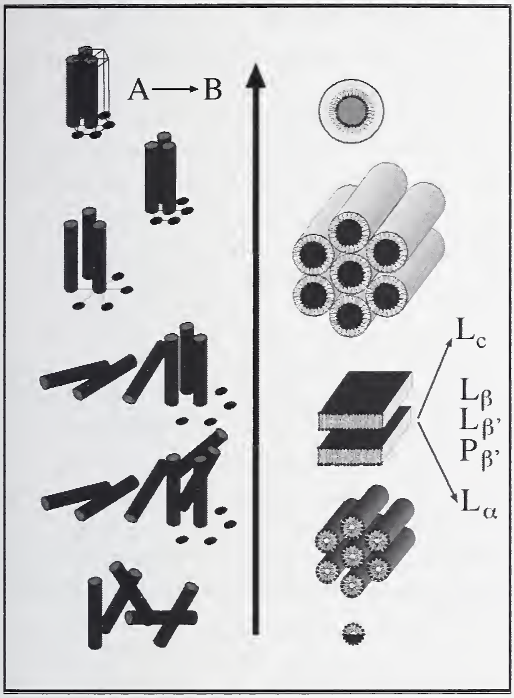

**FIGURE 29**

When mixed for gene transfection, DNA and lipid together assume structures that combine the organizing tendencies of pure DNA or pure lipid. With increasing concentration (read upward), DNA (left) progresses from a disorderly solution to ever more tightly packed hexagonal arrays of parallel double helices; even in dilute suspension, lipids form small micellar aggregates (bottom right), then cylindrical aggregates in hexagonal packing, followed by a variety of lamellar "L" structures. DNA can sit in the water between the lamellae as well as inside the tubes of the inside-out lipid arrays that occur at still higher concentrations. We measure the energies or work of creating DNA and lipid assemblies, extract the physical principles that drive molecular organization, then bring these ideas to the design of transfection strategies.

Our theoretical work has involved a fundamental reconsideration of van der Waals interactions as they act in liquids and solids. Most earlier work treated interacting bodies simplistically. For example, a membrane was treated as though its interface with surrounding water was a sudden step. We have recently generalized the theory so that the important polarizability of the material can be more realistically described as the spatially varying property that indeed it is. It was the ability to handle spatially varying properties that attracted our "computer-chip" collaborators. In a related theoretical formulation, we have developed formulae to compute interactions within multilayered systems. We are applying the formulae to measurements in a situation in which the behavior of membranes is driven by their proximity to confining surfaces. We can now model membrane surfaces more realistically, even those coated with polymers as used in drug-delivery preparations (see below).

### Polymer Stabilization of Drug-Delivery Lipids

*Cohen, Hansen, Podgornik*

From glycosylated cell surfaces to sterically stabilized liposomes, polymers attached to membranes attract biological and therapeutic interest. We are interested in determining to what extent the protective sponginess of polymers bound to membrane surfaces resembles the spongy character of polymers in free solution. We have found remarkably simple conditions under which the resemblance can be predicted and used in the design of polymer-stabilized membrane systems. We compared the forces between bilayers coated with polyethyleneglycol (PEG), obtained by our laboratory's osmotic stress method, with the osmotic pressure of PEG solutions. The criterion for a connection is that the polymer density and size in the "brush" on the membrane is high enough that in a bulk solution of equivalent weight concentration the polymer osmotic pressure is independent of polymer molecular weight. (Technically, the condition of molecular weight independence is referred to as the des Cloiseaux semi-dilute regime of bulk polymer solutions.)

In the design of liposomal drug-delivery systems, that is, drugs packaged into lipid-membrane vesicles, there is latitude to adjust the distance between PEGs grafted to the lipids as well as to choose PEG molecular weight. It is known that, to shield the membrane surface from proteins that might destroy the vesicle, the PEG density must be that of a "brush" ("brush" is a term used to describe the density of polymers attached to the surface such that the entire mass appears to have uniform concentration with no polymer-free space in between), i.e., the polymer is so dense that no other polymer can sneak through.

## SELECTED PUBLICATIONS

1. Hansen PL, Podgornik R, Parsegian VA. Osmotic properties of DNA: critical evaluation of counterion condensation theory. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics. 2001;64:021907.
2. Parsegian VA. Protein-water interactions. Int Rev Cytol. 2002;215:1-31.
3. Petrache HI, Zuckerman DM, Sachs JN, Killian JA, Koeppe RE, Woolf TB. Hydrophobic matching mechanism investigated by molecular dynamics simulations. Langmuir. 2002;18:1340-1351.
4. Podgornik R. Interactions and conformational fluctuations in macromolecular arrays. In: Holm C, Kekicheff P, Podgornik R, eds. Electrostatic effects in soft matter and biophysics. NATO Science Series II: Mathematics, Physics and Chemistry, volume 46, 2001.

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*Left NICHD April 2002.

# INTERMOLECULAR FORCES, RECOGNITION, AND DYNAMICS

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Our laboratory focuses on elucidating the coupling of forces, structure, and dynamics of biologically important macromolecules. The next challenge in structural biology is to understand the physics of interactions between molecules in aqueous solution. The ability to take advantage of the increasing number of protein and nucleic acid structures determined by x-ray crystallography and solution NMR will depend critically on this knowledge in order to understand the strength and specificity of interactions among biologically important macromolecules that control cellular function and to design agents rationally that can effectively compete with those specific interactions associated with disease. Our earlier results have shown that experimentally measured forces differ markedly from those predicted by current, conventionally accepted theories of intermolecular interactions. We have interpreted the observed forces as indicating a dominant contribution from water-structuring energetics. In our research, we use osmotic stress and x-ray scattering to measure directly forces between biological macromolecules in macroscopic condensed arrays. We also measure changes in hydration accompanying specific recognition reactions of biologically important macromolecules in dilute solution. Our research represents a first step toward linking our observation of dominating hydration forces between surfaces in condensed arrays to the interaction of individual molecules in solution.

## Direct Force Measurements

*Rau, in collaboration with Yang*

We investigated the role of small solutes in the precipitation of DNA by spermidine. Contrary to conventional expectations, we find that spermidine-induced assembly of DNA by alcohols is not facilitated by a lowered dielectric constant and increased electrostatic attraction but rather occurs because alcohols are excluded from DNA. Using the osmotic stress technique, we were able to map the spatial distribution function of two alcohols from the DNA surface by assessing the sensitivity of interhelical spacing as measured by x-ray diffraction to alcohol concentration. The concentration profile is exponential with a 3–4 Å decay length characteristic of a repulsive hydration force. Alcohols interact with DNA through their effect on water structuring. For homologous alcohols, the magnitude of the repulsion seems to scale with size. 2-Methyl-2,4-pentanediol is twice the size of i-propanol and is twice as well excluded. However, given that glycerol is intermediate in size between these two alcohols but is not observably excluded, size enters as an interaction summation over the chemical moieties of the solute rather than as a steric crowding.

We previously showed that the strong exclusion of salts and polar solutes from the nonpolar polymer hydroxypropyl cellulose is also attributable to hydration interactions. The strength and ubiquity of these interactions means that the thermodynamics of assembly and recognition reactions that occur within the crowded environment of a cell will differ dramatically from dilute solution.

The polar solute betaine glycine inhibits spermidine-induced assembly. In this case, we see an exponential increase in the concentration of betaine as helices move closer, once again with the 3–4 Å decay length characteristic of hydration forces. In contrast to alcohols, these are attractive rather than repulsive water-structuring forces. We can take advantage of this inclusion to probe DNA-DNA attractive forces. In essence, betaine applies outward pressure on the DNA assembly that is resisted by spermidine-mediated attraction. Most surprisingly, we find a transition to a much weaker attractive state as betaine concentration increases. We have long suspected that DNA assembly is accompanied by a loss in entropy as bound counterions are localized to maximize attraction. Indeed, the interplay between attractive forces and motional constraints or ordering is a common feature of many recognition reactions. We are currently investigating if the transition is caused by an abrupt change from correlated or ordered binding of spermidine to an uncorrelated or disordered binding. We are now planning experiments to determine the energies and entropies involved with this process.

### Water and Recognition Reactions

*Sidorova, Rau*

We have continued investigating the role of water in the sequence-specific recognition reaction of the restriction nuclease EcoRI. The exceptionally stringent specificity of this enzyme is a paradigm for recognition reactions. We measure the number of waters coupled to a binding reaction by assessing the dependence of the binding free energy on water chemical potential or, equivalently, osmotic pressure. We previously found that a nonspecific complex of the protein sequesters about 110 more waters than the specific one and that this water is probably at the protein-DNA interface. We have further shown that the dissociation rate of the specific complex is highly sensitive to osmotic pressure, requiring the net binding of 120 to 150 waters. During this past year, we investigated the temperature sensitivity of the ratio of specific and nonspecific binding constants, measured directly by using a competition assay. We found that the difference of 110 waters between the two complexes is temperature-independent. We further found that the heat capacity change between specific and nonspecific binding is only about half of that found for specific binding alone. Since changes in heat capacity in binding reactions are generally ascribed to the release of structured water, the water trapped at the protein-DNA interface of the nonspecific complex is less likely to be ordered than the water bound to the two isolated surfaces.

At low osmotic stresses, 110 sequestered waters are found even for complexes with noncognate sequences that differ by only a single base pair from the specific recognition sequence. At least some of this water, however, can be removed from the noncognate complexes by applying sufficiently high osmotic pressures. Given that equilibration times at high stresses are too long for convenient measurement of binding constants, we have used dissociation rates to determine water loss. We are able to remove water only from those complexes with sequences that differ by one base pair. The amount of water we are able to remove from a noncognate complex correlates with the enzyme's ability to cleave the sequence. Water and function seem closely connected.

We are beginning to investigate the correlation between water and binding strength for DNA-Cro protein complexes. Recognition stringency for this repressor is more typical of specific sequence DNA-protein binding systems. Single base-pair changes from the optimal recognition sequence reduce binding energies by only a few kT.

## SELECTED PUBLICATIONS

1. Bonnet-Gonnet C, Leikin S, Chi S, Rau DC, Parsegian VA. Measurement of forces between hydroxypropylcellulose polymers: temperature favored assembly and salt exclusion. J Phys Chem. 2001;105:1877-1886.

2. Sidorova NY, Rau DC. Linkage of EcoRI dissociation from its specific DNA recognition site to water activity, salt concentration, and pH: separating their roles in specific and nonspecific binding. J Mol Biol. 2001;310:801-816.

## COLLABORATORS

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# BIOPHYSICS OF LARGE MEMBRANE CHANNELS

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We focus on channel-facilitated transport of metabolites and other large solutes across cell and organelle membranes. Membrane proteins are sensitive to their immediate environment, including, for example, surrounding lipids and local acidity. Therefore, to study channels under precisely controlled conditions, we reconstitute channel proteins into planar lipid bilayers. The channels include VDAC (Voltage-Dependent Anionic Channel from the outer membrane of mitochondria), OmpF (general bacterial porin), LamB (sugar-specific bacterial porin), alpha-hemolysin (channel formed in the target cell membranes by a toxin from *Staphylococcus aureus*), alamethicin (oligomeric peptide channel), and syringomycin E (oligomeric lipopeptide channel). All have aqueous pores of 1 nm or larger diameter.

Emerging evidence demonstrates that rather than being merely "general diffusion pores," most of the large channels are structures with a high specialization for the solutes they allow to cross membranes. The main goal of our unit is to unveil the main physical principles of channel selectivity to solutes other than small ions. Such knowledge will advance our understanding of the molecular mechanisms responsible for metabolite flux regulation under normal conditions and in pathology.

## Channel-Facilitated Solute Transport Studied at the Single-Channel Level: "Molecular Coulter-Counting"

*Komarov, Nestorovich, Rostovtseva, Bezrukov; in collaboration with Colombini, Danelon, Kullman, Winterhalter*

Movement of solutes (mono- and dinucleotides, sugars, antibiotics) through bacterial and mitochondrial porins induces current fluctuations that are readable in terms of the molecular dynamics of solute transport. Large channels seem to be designed by nature to serve a different purpose from that of the small channels known to neurophysiologists, i.e., they are designed to regulate transport of molecular species that are larger than halide or metal ions. The large channels are pathways for metabolites and macromolecules such as proteins and nucleic acids, even though they can pass small ions. Measurement of the current carried by the small ions permits access not only to the structural changes in the channel molecule resulting from processes such as voltage or ligand gating but also to the transport of larger molecules through their transient occlusion of small-ion current.

This year, we reported the main results of our studies of maltoporin-facilitated sugar transport. We have shown that single-channel experiments offer new possibilities for revealing the molecular details of the interaction between the metabolite and the channel. The maltoporin channel (LamB) is asymmetric and, when added from one side of the membrane only, predominantly inserts in an oriented manner. By analyzing time-resolved transient interruptions in the maltoporin ionic current in the presence of different-sized maltodextrins, we find that only one sugar molecule is required to block one of the pores completely in the maltoporin trimer. The probability of simultaneous blockage of different pores increases with sugar concentration in a manner that demonstrates their mutual independence. The asymmetry of the channel structure manifests itself in two ways. First, it is seen as an asymmetric response to applied voltage under otherwise symmetric conditions and, second, as asymmetric rates of sugar entry into the channel with asymmetric (one-sided) sugar addition. Importantly, we find that the sugar residence time in the pore does not depend on which side the sugar is added. This voltage-dependent time is the same for either symmetric, *cis*, or *trans* sugar addition. This observation suggests that once a sugar molecule is captured by the greasy slide of the channel structure, it spends enough time there to "forget" by which entrance it was captured. This also means that the blockage events studied in our work represent sugar translocation events and not just binding at and release from the same entrance of the channel.

In a study with mitochondrial porin VDAC, we have shown that the dynamics of nucleotides inside the channel are sensitive not only to the nucleotide size and net charge but also to its three-dimensional structure. We also find that VDAC with pore radius of 1.2 to 1.5 nm can select among comparably charged molecules with a much smaller effective radius, 0.4 to 0.5 nm. The molecules under study were the nucleotides ATP, ADP, AMP, UTP, NAD, and NADH and the synthetic anions tetraglutamate (T-Glu) and 1-hydroxypyrene-3,6,8-trisulfonate (HPTS). All nucleotides caused a decrease in the conductance of VDAC in sodium chloride solution, showing that they could penetrate into the channel. In contrast, T-Glu and HPTS did not change the single-channel conductance, suggesting exclusion from the channel. We propose that the electrostatic environment within the VDAC aqueous pore has been evolutionally selected to favor the passage of nucleotides. Interestingly, the properties of solute-induced current fluctuations differ between nucleotides and synthetic anions as well as between different nucleotides, thus demonstrating that nucleotide dynamics within the pore depend on their detailed structure.

We have also extended our investigations of the mechanisms of antibiotic translocation through bacterial pores. It is well known that membrane permeability barriers are among the main reasons for bacterial antibiotic resistance. Using ion channel reconstitution and high-resolution conductance recording, we find that penicillins and cephalosporins are designed, either by targeting organisms or by pharmaceutical laboratories, to interact strongly with bacterial porin, OmpF. Thus, we demonstrate that, in analogy to substrate-specific channels that evolved to bind certain metabolite molecules, antibiotics have "evolved" to be channel-specific. The charge distribution of an efficient antibiotic complements the charge distribution at the narrowest part of the bacterial porin and, due to the resulting attraction, facilitates its translocation.

### Theory of Channel-Facilitated Solute Transport

*Bezrukov; in collaboration with Berezhkovskii, Pustovoit*

Accumulating evidence (see above) demonstrates that substrate-specific channels display pronounced binding to the corresponding substrates. We address the problem of solute translocation through membrane channels by using the Smoluchowski equation for diffusion in coordinate-dependent potential and with coordinate-dependent mobility. In a one-dimensional diffusion model with radiation boundary conditions obtained in our previous work, we give exact analytical expressions for the particle translocation probabilities. We also run Brownian dynamics simulations to verify the model and the quantitative predictions of our theory. In this theoretical study, we rationalize multiple observations of substrate-channel binding by showing that a potential well inside the channel is able to increase greatly the transit probability of the particle through the channel.

### Syringomycin E Channel: a Lipidic Pore Stabilized by Lipopeptide

*Gurnev, Malev, Nestorovich, Schagina, Takemoto, Bezrukov*

Highly reproducible ion channels of the lipopeptide antibiotic syringomycin E (SRE) demonstrate unprecedented involvement of the host bilayer lipids. We find that, in addition to a pronounced influence of lipid species on the open-channel ionic conductance, the membrane lipids play a crucial role in channel gating. The lipid charge and lipid dipole moment modify the effective gating charge, which characterizes sensitivity of the conformational equilibrium of the SRE channels to the transmembrane voltage. We show that the type of host lipid determines both the absolute value and the sign of the gating charge. With negatively charged bilayers, the gating charge sign inverts with increased salt concentration or decreased pH. We also demonstrate that the replacement of lamellar by non-lamellar lipid inhibits channel formation. These observations suggest that the asymmetric channel directly incorporates lipids. The charges and dipoles resulting from the structural inclusion of lipids are important determinants of the overall energetics that underlie channel gating. We conclude that the SRE channel may serve as a biophysical model to link studies of ion channels with those of lipidic pores in membrane fusion.

## SELECTED PUBLICATIONS

1. Berezhkovskii AM, Pustovoit MA, Bezrukov SM. Channel-facilitated membrane transport: transit probability and interaction with the channel. J Chem Phys. 2002;116:9952-9956.
2. Berezhkovskii AM, Pustovoit MA, Bezrukov SM. Effect of binding on particle number fluctuations in a membrane channel. J Chem Phys. 2002;116:6216-6220.
3. Kullman L, Winterhalter M, Bezrukov SM. Transport of maltodextrins through maltoporin: a single-channel study. Biophys J. 2002;82:803-812.
4. Malev VV, Schagina LV, Gurnev PA, Takemoto JY, Nestorovich EM, Bezrukov SM. Syringomycin E channel: a lipidic pore stabilized by lipopeptide? Biophys J. 2002;82:1985-1994.
5. Nestorovich EM, Danelon C, Winterhalter M, Bezrukov SM. Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. Proc Natl Acad Sci USA. 2002;99:9789-9794.
6. Rostovtseva TK, Komarov A, Bezrukov SM, Colombini M. Dynamics of nucleotides in VDAC channels: structure-specific noise generation. Biophys J. 2002;82:193-205.
7. Rostovtseva TK, Komarov A, Bezrukov SM, Colombini M. VDAC channels differentiate between natural metabolites and synthetic molecules. J Membrane Biol. 2002;187:147-156.
8. Rostovtseva TK, Nestorovich EM, Bezrukov SM. Partitioning of differently sized poly(ethylene glycol)s into OmpF porin. Biophys J. 2002;82:160-169.

## COLLABORATORS

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# THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS: ITS ENDOCRINE DISORDERS AND ITS INTERACTIONS WITH THE IMMUNE SYSTEM

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We investigate the molecular basis for genetic disorders of the hypothalamic-pituitary-adrenal (HPA) axis, such as pituitary tumors, adrenal insufficiency, ACTH resistance, congenital adrenal hyperplasia (CAH), glucocorticoid resistance/hypersensitivity, and mineralocorticoid resistance; diseases of the hypothalamic-pituitary-gonadal (HPG) axis, such as hypothalamic hypogonadism; and metabolic and immune disorders related to the functions of the HPA and HPG axes and the autonomic system, such as premature adrenarche, eating disorders that include anorexia and bulimia nervosa and adolescent obesity, childhood asthma and rheumatoid arthritis, the premenstrual tension syndrome, and postpartum and climacteric depression and autoimmunity. We also study the critical influences of early life stress on the later development of behavioral disorders (dysthymia, depression, dissociative and conduct disorders, substance abuse) and metabolic disorders (metabolic syndrome X). Thus, by identifying pathogenetic defects of the adrenocorticotropic hormone (ACTH) and glucocorticoid receptor genes and of genes of related molecules, the section has elucidated the molecular mechanisms of the hereditary diseases, respectively, congenital isolated glucocorticoid deficiency and glucocorticoid resistance syndromes and of acquired states such as AIDS-related glucocorticoid hypersensitivity (lipodystrophy and insulin resistance) syndrome. We also conduct research to increase our understanding of the interactions between the endocrine and immune systems in both experimental animals and humans.

## A Natural Determinant of Glucocorticoid Sensitivity in Human Tissues and Its Role in Human Pathophysiology

*Chrousos, Kino, De Martino, Charmandari, Souvatzoglou, Vottero, Alesci*

The glucocorticoid receptor gene, located in the long arm of chromosome 5, encodes two proteins produced by alternative splicing: the classic glucocorticoid receptor, designated GRalpha, and a nonligand-binding variant called GRbeta. We demonstrated that both isoforms are expressed in human tissues and that the nonligand-binding receptor is present at high concentrations in normal human tissues and exerts dominant negative effects on the classic receptor. Given that alteration in glucocorticoid receptor gene splicing would produce different ratios of GRalpha to GRbeta, it is possible to envisage that the ratio of glucocorticoid receptor types regulates tissue sensitivity to glucocorticoids. A change in sensitivity might have major implications for human pathophysiology in that both glucocorticoid resistance and glucocorticoid hypersensitivity could produce disease states. The former causes hyperandrogenism and hypertension while the latter could be involved in metabolic disorders such as visceral obesity or syndrome X, affective disorders such as depression, and immune disorders characterized by suppression of the inflammatory/immune response and vulnerability to infectious diseases and/or tumors. We showed that glucocorticoid-resistant asthma type II is associated with a major shift in the GRalpha-to-GRbeta ratio toward GRbeta predominance, explaining the profound resistance of patients' cells to glucocorticoids. We observed a similar shift in cells from glucocorticoid-resistant New World monkeys. We demonstrated that normal human neutrophils, which are naturally glucocorticoid-resistant, also over-express GRbeta. Recently, we showed how the GRbeta isoform causes glucocorticoid insensitivity. The isoform forms heterodimers with GRalpha, which then inhibits interaction with the nuclear receptor p160 coactivators' activation of glucocorticoid receptor gene transcription.

### Viral Determinants of Glucocorticoid Hypersensitivity in Patients with AIDS and AIDS-Related Lipodystrophy and Insulin Resistance Syndrome

*Chrousos, Kino, De Martino, Charmandari, Souvatzoglou, Vottero, Alesci*

Recently, we described a human example of a glucocorticoid hypersensitivity state: one of the accessory proteins of the HIV-1 called Vpr acts as a co-regulator of the glucocorticoid receptor, altering the sensitivity of human cells to glucocorticoids so that viral replication is favored. Such hypersensitivity explains several manifestations of AIDS, including T helper 1 immunosupression, T helper 2 shift, myopathy and muscle atrophy, and visceral obesity-related insulin resistance with its metabolic and cardiovascular manifestations. Researchers found that Vpr coactivates the glucocorticoid receptor by binding to it and to the cointegrator molecule p300/CBP and by stabilizing the transcription initiation complex. Recently, we identified a second HIV-1 accessory protein called Tat that also acts as a co-activator of the glucocorticoid receptor. This protein binds to nuclear receptor p160 coactivators and cooperates with Vpr in potentiating the activity of glucocorticoids on transcription of responsive genes. It appears that the HIV-1 LTR promoter uses p300/CBP and p160 co-activators for its own activation as well. The ability of HIV-1 proteins like Vpr to influence the activity of nuclear receptor co-activators extends beyond the glucocorticoid signaling system. We identified the PPAR-gamma and the insulin systems as targets of Vpr in the host cell. Indeed, Vpr causes resistance to PPAR-gamma and insulin by directly interfering with the actions of insulin-sensitivity–promoting lipids and the fork-head transcription factor.

### Familial and Sporadic Glucocorticoid Resistance

*Chrousos, Kino, De Martino, Charmandari, Souvatzoglou, Vottero, Alesci*

In addition, our group recently analyzed patients with the familial/sporadic glucocorticoid resistance syndrome, which is caused by mutations of the glucocorticoid receptor gene. Two novel glucocorticoid receptor mutations, one replacing isoleucine (I) 559 with aspartic acid (N) and another in which isoleucine (I) 747 was replaced by methionine (M), both caused glucocorticoid resistance in the heterozygote state, acting in a transdominant negative fashion by forming function-deficient dimers with wild-type GRalpha. We examined the cause of the transdominant activity of these mutant receptors and found that GRI559N interferes with the translocation of wild-type GRalpha from the cytoplasm into the nucleus while GRI747M prevents proper interaction of the wild-type receptor with the p160 nuclear receptor co-activators. Recently, we studied a newborn female infant with severe sexual ambiguity, a novel presentation of the glucocorticoid resistance syndrome. In this patient, the GRalpha gene bore a mutation that increased its affinity for glucocorticoids and its ability to activate glucocorticoid-responsive genes.

### Pathophysiology and Therapy of Adrenal Hyperfunction

*Chrousos, Merke, Charmandari, Hiroi*

The goal of our research is to understand the mechanisms involved in diseases caused by adrenal hyperandrogenism. We use insights into the mechanism to generate hypotheses for new approaches to treatment. Deborah Merke is principal investigator for a clinical trial that is testing a new medical treatment for children with classic congenital adrenal hyperplasia (CAH). Preliminary results based on two years of therapy showed that children receiving the new treatment regimen had normalization of growth velocity and bone maturation. Thus, the regimen represents a promising novel treatment approach for children with classic 21-hydroxylase deficiency. Reproductive, metabolic, and other morbidities, including risk of tumor formation, particularly testicular tumors, are currently under investigation in the severe forms of the disease. Bilateral adrenalectomy performed on a severe case revealed massive lymphocytic infiltration and histologic signs of adrenal carcinoma, leading to new insights regarding lymphocyte-adrenal-cell interaction and adrenocortical tumor formation.

Recent clinical studies led to the novel finding of adrenomedullary dysplasia and hypofunction in patients with CAH. The results have important clinical implications. The protective function of the adrenal medulla may be absent in the most severe cases. Studies of hypoglycemia and exercise tolerance in patients are under way. It appears that the lack of a proper epinephrine response to exercise prevents an appropriate glucose response in CAH. Recently, children with CAH were found to be resistant to insulin, a novel finding with negative repercussions for these patients. Studies of healthy children are also under way, and preliminary results reveal sex- and age-differences in catecholamine secretion, a novel finding. New clinical initiatives include studies of the pathophysiology of premature adrenarche and the effects of androgens and other hormones on the brain. Psychological testing and head MRI studies are being conducted under way with children suffering from endocrine disorders, including CAH, Cushing's syndrome, and familial male precocious puberty. The studies will provide insight into hormonal influences on the brain during growth and development.

### Role of CRH in Behavior and Behavioral/Developmental/Sleep Disorders

*Chrousos, Charmandari; in collaboration with Cizza, Gold, Deuster, Susman, Dorn*

The group has used corticotropin releasing hormone (CRH) successfully to probe central mechanisms in patients with atypical, seasonal depression, the chronic fatigue/fibromyalgia syndromes, and the postpartum blues/depression syndromes. The patients' responses to CRH are indicative of mild, sustained hypocortisolism of central nervous system etiology, suggesting that a spectrum of diseases exists that are characterized by hypofunction of the central stress system. These syndromes contrast with melancholic depression, panic anxiety, and anorexia nervosa, for which we earlier demonstrated hyperactivity of the CRH system. As we have shown, the behavioral similarities between patients with Cushing's syndrome and atypical depression probably stem from a similar pathologic decrease in CRH secretion. Recently, we demonstrated that preadolescent and adolescent girls with a history of sexual abuse exhibited an HPA axis response to CRH similar to that of patients with melancholic depression or recovering anorexics. The findings suggest that major stress in children may have long-term effects on the HPA axis accompanied by effects on the psyche. Indeed, we found dysthymia or depression in all the sexually abused children studied. In a social nonhuman primate model, we demonstrated effects of parental abuse not only on the HPA axis but also on growth.

Individuals who are carriers of a 21-hydroxylase deficiency, such as parents of patients with CAH, are presumably totally asymptomatic. We studied the mood and HPA axis of such subjects, testing the hypothesis that a compensatory elevation of CRH secretion would be associated with indices of increased anxiety. We found that, indeed, compensatory changes in the activity of the HPA axis are apparent in a standard CRH test and that these changes correlate positively with anxiety. As a group, the carriers had higher anxiety scores than appropriate controls.

Studies in healthy normal volunteers demonstrated that, in middle age, the sleep centers in the brain are sensitized to the activational effects of CRH and glucocorticoids, a finding that explains the increasing vulnerability to stress- or caffeine-induced insomnia with advancing age. Studies in young and middle-aged patients with idiopathic insomnia showed that such patients have significant evening activation of their HPA axis and an overall activation of the axis that correlates positively with indices of poor quality sleep, such as decrements in amount of deep sleep and increments in wakefulness.

We also recently showed that antalarmin, a nonpeptide CRH antagonist that crosses the blood barrier, inhibits the stress-mimicking properties of CRH, including its effects on the HPA axis and behavior. This drug thus represents a new class of pharmacologic agents for the potential treatment of diseases characterized by CRH hypersecretion, such as melancholic depression, chronic anxiety disorder, anorexia nervosa, and idiopathic insomnia.

### Cytokines and the Inflammatory Response in Endocrine Hyper- and Hypo-Secretion States

*Chrousos; in collaboration with Elenkov, Gold, Sternberg*

Several cytokines, including interleukin 1, tumor necrosis factor-alpha (TNF-alpha), and interleukin (IL)-6, as well as several lipid mediators of inflammation, including platelet-activating factor and several eicosanoids, cause profound stimulation of the HPA axis, primarily by activating the CRH neuron and, through it, by suppressing and restraining the inflammatory/immune response. We recently studied IL-6 in humans and found that this cytokine is extremely potent in activating the HPA axis and that, at high doses, it causes systemic vasopressin secretion. At all doses, it is not noxious, unlike TNF-alpha and interleukin-1, which cause hypotension and vascular leakage. The group demonstrated that IL-6 is a potent stimulator of ACTH release in normal subjects and that catecholamines and glucocorticoids regulate IL-6 secretion. IL-6 levels are normal in patients with Cushing's syndrome but increase markedly after the surgical cure of hypercortisolism. The rebound elevation in circulating IL-6 levels immediately after surgical cure of hypercortisolism may be the cause of the systemic symptoms typical of the glucocorticoid withdrawal syndrome in these patients. Thus, IL-6 participates in an immune-endocrine feedback loop involving CRH, ACTH, and cortisol. We found that the stress hormones glucocorticoids and catecholamines inhibit the secretion of interleukin-12 and stimulate the secretion of IL-10 by monocytes/macrophages, leading to a shift from T helper 1– to T helper 2–directed immunity. We discovered that, in the transition from pregnancy to the postpartum period, the hormonal changes that take place cause a T helper 2 to T helper 1 shift, which explains the vulnerability of postpartum women to autoimmune disorders of the T helper 1 type, such as Hashimoto thyroiditis, multiple sclerosis, and rheumatoid arthritis.

### Leptin and Cytokines in Obesity, Polycystic Ovary Syndrome, and Sleep Disorders

*Chrousos, Ayala, Zoumakis, Bornstein; in collaboration with Vgontzas*

We recently demonstrated that, in addition to leptin, human fat secretes large amounts of TNF-alpha and Il-6 and that the secretion of adipose tissue cytokines is regulated by food intake and by the sympathetic nervous system. The secretion of these cytokines, e.g., Il-6, has a circadian rhythm that is influenced by sleep while circulating levels increase in proportion to the BMI and are further elevated by visceral adiposity. Sleep deprivation is associated with elevations of daytime IL-6, whose levels correlate with somnolence and fatigue. This is true for patients with insomnia who, during the day, have elevated levels not only of cortisol but also of IL-6. Interestingly, IL-6 concentrations in plasma are proportional to body fat, and viscerally obese subjects have further elevations that correlate with sleep apnea measures. Women with the polycystic ovary syndrome exhibit high cytokine and leptin levels and are at markedly increased risk for sleep apnea while girls with premature adrenarche show features of visceral adiposity such as high fasting leptin and insulin, predictors of later development of polycystic ovary syndrome and sleep apnea.

**Immune and Reproductive CRH and Role of CRH Antagonists**

*Chrousos, Zoumakis, Kabbani, Gravanis; in collaboration with Gold, Dorn*

The group demonstrated that CRH is produced locally at sites of inflammation and has profound pro-inflammatory effects at an autocrine/paracrine level. CRH is a potent degranulator of mast cells, a phenomenon that can be inhibited by antalarmin, the nonpeptide CRH antagonist specific for type 1 receptors. The antagonist has marked systemic anti-inflammatory actions and ameliorated the disease in adjuvant-induced arthritis, a model of rheumatoid arthritis, suggesting that CRH antagonists may be useful in the treatment of inflammatory disorders in man. CRH was also found in the ovary and endometrium, where it may participate in the inflammatory phenomena of ovulation, luteolysis, blastocyst implantation, and menstruation. Antalarmin blocked implantation in rats and labor in sheep, suggesting that CRH antagonists may have clinical applications in reproductive medicine.

## SELECTED PUBLICATIONS

1. Abad V, Chrousos GP, Reynolds J, Nieman L, Hill S, Weinstein R, Leong G. Glucocorticoid excess during adolescence leads to a major, persistent deficit in bone mass and increase in central body fat. J Bone Min Res. 2001;16:1879-1885.
2. Alesci S, Ramsey WJ, Bornstein SR, Chrousos GP, Hornsby PJ, Benvega S, Trimarchi F, Ehrhart-Bornstein M. Adenoviral vectors can impair adrenocortical steroidogenesis: clinical implications for natural infections and gene therapy. Proc Natl Acad Sci USA. 2002;99:7484-7489.
3. Carter CS, Altemus M, Chrousos GP. Neuroendocrine and emotional changes in the post-partum period. Progr Brain Res. 2001;133:241-249.
4. Charmandari E, Eisenhofer G, Mehlinger S, Carlson A, Wesley R, Keil M, Chrousos GP, New MI, Merke DP. Adrenomedullary function may predict phenotype and genotype in classic 21-hydroxylase deficiency. J Clin Endocrinol Metab. 2002;87:3031-3037.
5. Charmandari E, Weise M, Bornstein SR, Eisenhofer G, Keil MF, Chrousos G, Merke DP. Children with classic congenital adrenal hyperplasia have elevated serum leptin concentrations and insulin resistance. J Clin Endocrinol Metab. 2002;87:2139-2143.
6. Cizza G, Dorn LD, Lotsikas A, Sereika S, Rotenstein D, Chrousos GP. Circulating plasma leptin and IGF-1 levels in girls with premature adrenarche: potential implications of a preliminary study. Horm Metab Res. 2001;33:138-143.
7. Elenkov IJ, Wilder RL, Bakalov VK, Link AA, Dimitrov M, Fisher S, Crane M, Kanik K, Chrousos GP. Interleukin 12, tumor necrosis factor-alpha and hormonal changes during late pregnancy and early postpartum: implications for autoimmune disease activity during these times. J Clin Endocrinol Metab. 2001;86:4933-4938.
8. Erickson K, Thorsen P, Chrousos G, Grigoriadis DE, Khongsaly O, McGregor J, Schulkin J. Preterm birth associated neuroendocrine, medical, and behavioral risk factors. J Clin Endocrinol Metab. 2001;86:2544-2552.
9. Franchimont D, Galon J, Vacchio MS, Fan S, Visconti R, Frucht DM, Geenen V, Chrousos GP, Ashwell JD, O'Shea JJ. Positive effects of glucocorticoids on T cell development and function by upregulation of interleukin-7 receptor α. J Immunol. 2002;168:2212-2218.
10. Gabry KE, Chrousos GP, Rice KC, Mostafa RM, Sternberg E, Negrao AB, Webster EL, McCann SM, Gold PW. Marked suppression of gastric ulcerogenesis and intestinal responses to stress by a novel class of drugs. Mol Psych. 2002;7:474-483.
11. Galon J, Franchimont D, Hiroi N, Boettner A, Ehrhart-Bornstein M, Chrousos GP, Bornstein S. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J. 2002;16:61-71.
12. Ghizzoni L, Barreca A, Mastorakos G, Furlini M, Vottero A, Ferrari B, Chrousos GP, Bernasconi S. Leptin inhibits steroid biosynthesis by human granulosa-lutein cells. Horm Metab Res. 2001;33:323-328.
13. Gold PW, Chrousos GP. Organization of the stress system and its dysregulation in melancholic and atypical depression: high *vs.* low CRH/NE states. Mol Psychiatry. 2002;7:254-275.

14. Gold PW. Gabry KE, Yasuda MR. Chrousos GP. Divergent endocrine abnormalities in melancholic and atypical depression: clinical and pathophysiologic implications. Endocrinol Metab Clin North Am. 2002;31:37-62.

15. Habib K. Gold PW. Chrousos G. Hypothalamic-pituitary-adrenal axis: introduction to physiology and pathophysiology. Horm Brain Behav. 2002;4:841-866.

16. Habib K. Gold PW. Chrousos GP. Neuroendocrinology of stress. Endocrinol Metab Clin North Am. 2001:30:695-728.

17. Hiroi N. Chrousos GP, Kohn B, Lafferty A, Abu-Asab M, Bonat S, White A, Bornstein SR. Adrenocortical-pituitary hybrid tumor causing Cushing's syndrome. J Clin Endocrinol Metab. 2001;86:2631-2637.

18. Hiroi N. Wong ML, Licinio J, Park C, Young M, Gold PW, Chrousos GP. Bornstein SR. Expression of corticotropin releasing hormone receptors type I and type II mRNA in suicide victims and controls. Mol Psychiatry. 2001:6:540-546.

19. Kalandaridou S, Chrousos GP. Clinical review 148. Monogenic disorders of puberty. J Clin Endocrinol Metab. 2002:87:2481-2494.

20. Kino T. Gragerov A. Slobodskaya O, Tsopanomihalou-Nglotsou M. Chrousos GP, Pavlakis G. Human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr induces transcription of the HIV-1 and glucocorticoid-responsive promoters by binding directly to p300/CBP coactivators. J Virol. 2002:76:9724-9734.

21. Kino T. Slobodskaya O. Pavlakis N, Chrousos GP. Nuclear receptor coactivator p160 proteins enhance the human immunodeficiency virus (HIV) type 1 long terminal repeat promoter by bridging promoter bound factors and the Tat/pTEF² complex. J Biol Chem. 2002:277:2396-2405.

22. Kino T, Stauber RH, Resau JH, Pavlakis G, Chrousos GP. Pathologic human glucocorticoid receptor (GR) mutant has a transdominant negative effect on the wild type GR by inhibiting its translocation into the nucleus: importance of the ligand-binding domain for intracellular GR trafficking. J Clin Endocrinol Metab. 2001;86:5600-5608.

23. Koch CA. Chrousos GP. Chandra R. Evangelista RS. Gilbert JC. Nobuhara K. Zhuang Z, Vortmeyer AO. Two-hit model for tumorigenesis of nevoid basal cell carcinoma (Gorlin) syndrome-associated hepatic mesenchymal tumor. Am J Med Genet. 2002;109:74-76.

24. Kohno M. Kawahito Y. Tsubouchi Y, Hashiramoto A, Yamada R, Inoue KI, Kusaka Y, Kubo T, Elenkov IJ, Chrousos GP. Kondo M. Sano H. Urocortin expression in synovium of patients with rheumatoid arthritis and osteoarthritis: relation to inflammatory activity. J Clin Endocrinol Metab. 2001;86:4344-4352.

25. Kraft M, Hamid Q, Chrousos GP, Martin RJ, Leung DY. Decreased steroid responsiveness at night in nocturnal asthma. Is the macrophage responsible? Am J Respir Crit Care Med. 2001:163:1219-1225.

26. Kuperman H. Damiani D. Chrousos GP. Dichtchekenian V. Manna TD. Filho VO, Setian N. Evaluation of the hypothalamic-pituitary-adrenal axis in children with leukemia before and after 6 weeks of high-dose glucocorticoid therapy. J Clin Endocrinol Metab. 2001;86:2993-2996.

27. Makrigiannakis A. Zoumakis E. Kalandaridou S. Coutifaris C. Coukos G. Rice K. Gravanis A. Chrousos GP. Corticotropin-releasing hormone (CRH) promotes blastocyst implantation and early maternal tolerance. Nat Immunol. 2001;2:1018-1024.

28. Maurer M. Trajanoski Z. Hiroi N. Frey G. Galom J. Willenberg HS. Gold PW. Chrousos GP, Scherbaum WA. Bornstein SR. Differential gene expression profile of glucocorticoids, testosterone and dehydroepiandrosterone in human cells. Horm Metab Res. 2001;33:691-695.

29. Meduri GU. Tolley EA. Chrousos GP. Stentz F. Prolonged methylprednisolone treatment suppresses systemic inflammation in patients with unresolving ARDS: evidence for inadequate endogenous glucocorticoid secretion and inflammation-induced immune cell resistance to glucocorticoids. Am J Resp Crit Care Med. 2002:165:983-991.

30. Mendonca BB. Leite MV. deCastro M. Kino T. Elias LL. Bachega TAS. Billerbeck AE. Arnhold IAJP. Chrousos GP. Latronico AC. Female pseudohermaphrodotism caused by a novel homozygous missence mutation of the glucocorticoid receptor gene. J Clin Endocrinol Metab. 2002;87:1805-1809.

31. Merke DP, Cho D, Calis KA, Keil MF, Chrousos GP. Hydrocortisone suspension and hydrocortisone tablets are not bioequivalent in the treatment of children with congenital adrenal hyperplasia. J Clin Endocrinol Metab. 2001;86:441-445.

32. Meyer SE, Chrousos GP, Gold PW. Major depression and the stress system: a life span perspective. Dev Psychopathol. 2001;13:565-580.

33. Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA, Hauner H. Human breast adipocytes express interleukin-6 (IL-6) and its receptor system increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. J Clin Endocrinol Metab. 2001;86:2281-2288.

34. Vgontzas AN, Bixler EO, Lin HM, Prolo P, Mastorakos G, Vela-Bueno A. Kales A, Chrousos GP. Chronic insomnia is associated with nyctohemeral activation of the hypothalamic-pituitary-adrenal axis clinical implications. J Clin Endocrinol Metab. 2001;86:3787-3794.

35. Vgontzas AN, Chrousos GP. Sleep, the hypothalamic-pituitary-adrenal axis. and cytokines: multiple interactions and disturbances in sleep disorders. Endocrinol Metab Clin North Am. 2002;31:15-36.

36. Vgontzas AN, Legro RS. Bixler EO, Grayev A, Kales A, Chrousos GP. Polycystic ovary syndrome is associated with obstructive sleep apnea and daytime sleepiness role of insulin resistance. J Clin Endocrinol Metab. 2001;86:517-520.

37. Vgontzas AN, Zoumakis M. Papanicolaou DA. Bixler E, Prolo P, Lin HM, Vela-Bueno A. Kales MD, Chrousos GP. Chronic insomnia is associated with a shift of IL-6 and TNFα secretion from nighttime to daytime. Metabolism. 2002;29:1252-1261.

38. Vottero A, Kino T, Combe H, Lecomte P, Chrousos GP. A novel, C-terminal dominant negative mutation of the glucocorticoid receptor causes familial glucocorticoid resistance through abnormal interactions with p160 steroid receptor coactivators. J Clin Endocrinol Metab. 2002;87:2658-2667.

39. Webster EL, Barrientos RM, Contoreggi C, Isaac MG, Ligier S, Habib K, Chrousos GP. McCarthy EF, Rice KC, Gold PW, Sternberg EM. Corticotropin-releasing hormone (CRH) antagonist attenuates adjuvant-induced arthritis: evidence supporting major role for CRH in peripheral inflammation. J Rheumatol. 2002;29:1252-1261.

40. Young IR, Chan EC, Smith R, Chrousos GP, Veldhuis JD, Canny BJ. The effect of antalarmin, a novel corticotropin releasing hormone antagonist on the dynamic function of the preterm ovine fetal hypothalamic-pituitary-adrenal axis. Neuroendocrinology. 2002;76:47-54.

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# DIAGNOSIS, PATHOPHYSIOLOGY, AND MOLECULAR BIOLOGY OF NEUROENDOCRINE TUMORS

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We are conducting patient-oriented, conceptually innovative research on the etiology, pathophysiology, diagnosis, prognosis, and treatment of pheochromocytoma and other neuroendocrine tumors. Projects include not only translational research, i.e., applying basic science knowledge to clinical diagnosis, pathophysiology, and treatment, but also "reverse translation research," in which appreciation of clinical findings leads to new concepts that basic researchers can pursue in the laboratory. The long-range goal is to develop new and improved approaches in the diagnosis and treatment of pheochromocytoma. As an initial step towards this goal, our studies focus on molecular and genetic mechanisms that may elucidate the basis for the predisposition to develop pheochromocytomas and for the expression of different neurochemical phenotypes and malignant potentials of these tumors. We also focus on new imaging approaches, based on 6-[$^{18}$F]fluorodopamine ([$^{18}$F]-6F-DA) positron emission tomographic (PET) scanning, and new biochemical diagnostic criteria, based on measurement of plasma metanephrines. Finally, our research team will be conducting clinical studies of the treatment of malignant pheochromocytoma using $^{131}$I-metaiodobenzylguanidine.

### Molecular Genetic Basis of Tumorigenesis in Familial and Sporadic Pheochromocytoma

*Brouwers, Koch,[a] Pacak; in collaboration with Eisenhofer, Zhuang, Linehan, Walther*

According to Knudson's well-established "two hit" hypothesis of hereditary tumor development, inactivation of both copies of a tumor suppressor gene is required to promote tumor growth. In contrast, the concept of tumorigenesis for oncogenes considers mutation in the respective oncogene on one allele to be sufficient. Our collaborators at NINDS recently proposed a new model of oncogene-driven tumor initiation. The model suggests that overrepresentation of the mutated *c-met* oncogene is an important mechanism of hereditary papillary renal cell tumorigenesis. Homology between *MET* and *RET* led us to hypothesize that tumors in patients with multiple endocrine tumors of the neoplasia type 2 (MEN 2) might develop by a similar mechanism. In this syndrome, which mainly manifests itself as pheochromocytoma and medullary thyroid carcinoma, patients with a *RET* germline mutation develop tumors as early as in the first month of life and as late as in the seventh decade. We found that duplication of the mutated *RET* allele in trisomy 10 represents a possible mechanism of tumorigenesis in MEN 2-associated pheochromocytomas. However, some of the pheochromocytomas did not reveal overrepresentation of mutated *RET*. Instead, these tumors exhibited loss of the wild-type *RET* allele, also leading to a dominant effect of mutant *RET*. We could also demonstrate the allelic imbalance between the mutant and wild-type *RET* allele in MEN 2-associated medullary thyroid carcinoma and in the TT cell line. In this cell line, we did not find trisomy 10 or loss of the wild-type *RET* allele as mechanisms of overrepresentation of mutant *RET* but, instead, a tandem duplication of *RET*, which constitutes a third mechanism for imbalance between mutant and wild-type *RET*. Some of the MEN 2–associated tumors, however, did not reveal mutant and wild-type *RET* imbalance. We therefore searched for other mechanisms of a "second hit" in these tumors and found somatic *VHL* gene deletion and mutation. In future studies of non-hereditary pheochromocytomas, we shall attempt to assess whether tumorigenesis of sporadic pheochromocytoma shares pathogenetic pathways with hereditary counterparts and to identify genes involved in tumorigenesis.

### Imaging Modalities in the Evaluation of Patients with Pheochromocytoma

*Pacak, Yu; in collaboration with Carrasquillo, Chen, Reynolds*

Pheochromocytoma constitutes a form of surgically curable hypertension, and failure to diagnose and localize the tumor can result in sudden, unexpected, and potentially lethal complications. Computed tomography and magnetic resonance imaging have good sensitivity but poor specificity, and commonly available nuclear imaging modalities, such as $^{131}$I-metaiodobenzylguanidine scintigraphy, have high specificity but limited sensitivity. Positron emission tomographic scanning uses short-lived positron-emitting radionuclides, which allows administration of large tracer doses, resulting in high count density and, thus, superior resolution compared with conventional single photon emitters used in nuclear medicine. 6-[$^{18}$F]-Fluorodopamine, a sympathoneural imaging agent developed within the NIH intramural research program, is a positron-emitting analog of dopamine. In catecholamine-synthesizing cells, including pheochromocytoma cells, both the plasma membrane norepinephrine transporter and intracellular vesicular monoamine transporter actively and avidly transport 6-[$^{18}$F]fluorodopamine. Our results show that, in patients with pheochromocytoma (including metastatic disease), 6-[$^{18}$F]-fluorodopamine PET scanning can detect and localize pheochromocytomas with high sensitivity. In patients in whom the diagnosis of pheochromocytoma is considered but excluded because of negative biochemical results, 6-[$^{18}$F]-fluorodopamine PET scans are consistently negative. Furthermore, our preliminary results show that 6-[$^{18}$F]-fluorodopamine is superior to $^{131}$I-metaiodobenzylguanidine for diagnostic localization of benign and metastatic pheochromocytoma.

### Mechanisms Linking Different Pheochromocytoma Tumor Cell Phenotypes and Clinical Presentation of Disease to Specific Underlying Mutations

*Pacak, Koch,[a] Brouwers; in collaboration with Zhuang, Eisenhofer*

Our recent results indicate that the neurochemical and histopathological phenotypic features of pheochromocytomas from patients with von-Hippel Lindau (VHL) syndrome are distinct from those from MEN 2 patients and are accompanied by differences in the clinical presentations of the disease. In MEN 2 patients with pheochromocytoma, there is a high incidence of paroxysmal attacks, and these patients exhibit more robust adrenergic and hemodynamic responses to glucagon than do VHL patients with pheochromocytoma. Pheochromocytomas from patients with VHL disease exhibit a distinctly noradrenergic phenotype, in which the tumor produces norepinephrine specifically, while those from patients with MEN 2 show an adrenergic phenotype, in which the tumor also produces epinephrine. These phenotypic variations result from differences in expression of certain genes, such as that encoding phenolethanolamine-N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine.

We will correlate the results of these studies and neurochemical analysis of tumor catecholamine content with the underlying germline and somatic mutations. Morphologic studies, in conjunction with genetic analysis, will attempt to separate these tumors into morphologically and genetically distinct subsets, with potential implications for the diagnosis and biologic behavior of pheochromocytomas in individual patients. Follow-up studies combining laser-capture microdissection with differential display or microarray analysis will trace phenotypic differences in tumors to underlying differences in gene expression and, ultimately, to the basic somatic or germline mutations responsible for the tumor.

### Chromaffin and Pheochromocytoma Cell Cultures

*Pacak, Brouwers, Perera, Kukura; in collaboration with Alesci, Eisenhofer*

There is no known effective treatment for malignant pheochromocytoma. Developing effective treatments requires a means to test new drugs for their ability to kill the tumor cells selectively. New therapeutic options, such as gene therapy and immunotherapy, should also be considered. This project is designed to establish human pheochromocytoma cell lines to be used as *in vitro* models for testing radio-therapeutic or chemotherapeutic compounds, genetic manipulations, or vaccines that could be applied to the treatment or prevention of metastatic pheochromocytoma and other tumors of neural crest origin. The general goal is to validate the feasibility and effectiveness of any of these therapeutic approaches in new clinical trials at the NIH. Several studies are being conducted including: (1) establishing human pheochromocytoma cell lines; (2) identifying potential radio-therapeutic or chemotherapeutic drugs that might bind to or be taken up by and concentrated in the cells, oncolytic viruses or viral vectors for gene therapy, or proteins expressed specifically in pheochromocytoma tumor cells; (3) determining the cytotoxic efficacy of the drugs or treatments; (4) verifying that the drugs or treatments are not toxic to other cell lines; (5) developing new clinical research protocols to test the efficacy of the identified potential treatments of pheochromocytoma, especially malignant or unresectable pheochromocytoma.

## SELECTED PUBLICATIONS

1. Eisenhofer G, Lenders JW, Pacak K. Choice of biochemical test for diagnosis of pheochromocytoma: validation of plasma metanephrines. Curr Hypertens Rep. 2002;4:250-255.
2. Eisenhofer G, Walther MM, Huynh T-T, Li S-T, Bornstein S, Mannelli M, Goldstein DS, Vortmeyer A, Lenders JWM, Linehan WM, Pacak K. Pheochromocytoma in von-Hippel Lindau syndrome and multiple endocrine neoplasia type 2 display distinct noradrenergic and adrenergic phenotype. J Clin Endocrinol Metab. 2001;86:1999-2008.
3. Koch CA, Huang SC, Moley JF, Azumi N, Chrousos GP, Gagel RF, Zhuang Z, Pacak K, Vortmeyer AO. Allelic imbalance of the mutant and wild-type *RET* allele in MEN 2-associated medullary thyroid carcinoma. Oncogene. 2001;20:7809-7811.
4. Koch CA, Huang SC, Zhuang Z, Stolle C, Azumi N, Chrousos GP, Vortmeyer AO, Pacak K. Somatic VHL gene deletion and point mutation in MEN 2A-associated pheochromocytoma. Oncogene. 2002;21:479-482.
5. Koch CA, Mauro D, Walther MM, Linehan,WM, Vortmeyer AO, Jaffe R, Pacak K, Chrousos GP, Zhuang Z, Lubensky IA. Pheochromocytoma in VHL disease: distinct histopathologic phenotype compared to pheochromocytoma in multiple endocrine neoplasia type 2. Endocr Pathol. 2002;13:17-27.
6. Koch CA, Vortmeyer AO, Diallo R, Poremba C, Giordano TJ, Sanders D, Bornstein SR, Chrousos GP, Pacak K. Survivin: a novel neuroendocrine marker for pheochromocytoma. Eur J Endocrinol. 2002;146:381-388.
7. Lenders JW, Pacak K, Walther MM, Linehan WM, Friberg P, Keiser HK, Goldstein DS, Eisenhofer G. Biochemical diagnosis of pheochromocytoma: which test is best? JAMA. 2002;287:1427-1434.
8. Pacak K, Eisenhofer G, Carrasquillo JA, Chen CC, Li ST, Goldstein DS. 6-[18F]fluorodopamine positron emission tomographic (PET) scanning for diagnostic localization of pheochromocytoma. Hypertension. 2001;38:6-8.
9. Pacak K, Fojo T, Goldstein DS, Eisenhofer G, Walther MM, Linehan WM, Bachemeicher L, Abraham J, Wood BJ. Radiofrequency ablation (RFA): a novel approach for the treatment of metastatic pheochromocytoma. J Natl Cancer Inst. 2001;93:648-649.

10. Pacak K, Goldstein DS, Doppman JL, Shulkin BL, Udelsman R, Eisenhofer G. A “pheo” lurks: novel approaches for localizing occult pheochromocytoma. J Clin Endocrinol Metab. 2001;86:3641-3646.
11. Pacak K, Linehan WM, Walther M, Goldstein DS, Eisenhofer G. Recent advances in genetics, diagnosis, localization, and treatment of pheochromocytoma. Ann Intern Med. 2001;134:114-129.
12. Pacak K, Tjurmina O, Palkovits M, Goldstein DS, Koch CA, Hoff T, Chrousos GP. Chronic hypercortisolemia inhibits dopaminergic activity in the nucleus accumbens but not the prefrontal cortex: an *in vivo* microdialysis study. Neuroendocrinology. 2002;76:148-157.

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# THE PHYSIOLOGY OF HYPERCORTISOLISM AND THE ENDOMETRIUM

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Over the past decade, we have made major contributions to the differential diagnosis of hypercortisolism. We established the corticotropin releasing hormone (CRH) test and inferior petrosal sinus sampling (IPSS) as major diagnostic tools in the identification of pituitary adenomas causing Cushing's syndrome. We investigate the biochemical and physiologic mechanisms of the reproductive cycle and pregnancy in women and the pathophysiology of endometriosis, leiomyomata, and infertility.

### Quality-of-Life Assessment in Cushing's Syndrome

*Nieman; in collaboration with Nansel*

We are now exploring quality-of-life issues in patients before and after successful treatment of Cushing's syndrome. We use the entire SF-36 questionnaire developed by John Ware and colleagues; the questionnaire has been validated for use in healthy individuals and various disease states. Patients also complete a symptom checklist and a questionnaire about recurrence, satisfaction with treatment, current treatment, and demographics. Initial analysis suggests that patients with active Cushing's syndrome have great impairment in physical activities and cognitive function and limit their social activities. They also demonstrate significant but less profound abnormalities in their sense of well-being and general health perception compared with the general population. Preliminary evaluation of patients after treatment suggests improvement in these areas, but some patients do not regain scores similar to the general population for as long as five years after treatment, suggesting significant long-term impairment of quality of life in some individuals.

### Salivary Cortisol for the Detection of Cushing's Syndrome

*Nieman; in collaboration with Papanicolaou, Mullen*

Clinical features such as weight gain, depression, hypertension, and menstrual irregularities, while common in the general population, may raise the possibility of Cushing's syndrome. Up to 30 percent of urine cortisol and dexamethasone suppression screening tests may return an incorrect result, suggesting that better tests are needed. We recently evaluated the utility of nighttime salivary cortisol measurement as a screening test for Cushing's syndrome. We evaluated 136 inpatients and four outpatients with possible Cushing's syndrome, 16 inpatients and seven outpatients with other non-adrenal disorders, and 34 healthy outpatients. Using cutpoints that excluded all subjects without Cushing's syndrome, we compared the sensitivity of nighttime salivary cortisol levels

(11:30 p.m. and midnight for inpatients and bedtime for outpatients), simultaneous inpatient serum cortisol levels, and urine glucocorticoid excretion for the detection of Cushing's syndrome. An assay-specific inpatient midnight or outpatient bedtime salivary cortisol of greater than 550 ng/dL (15.2 nmol/L) identified 93 percent of patients with Cushing's syndrome (CI: 89–98 percent) and excluded all individuals without the disorder. Salivary cortisol measurements worked as well as plasma measurements and better than urine glucocorticoid excretion in diagnosing Cushing's syndrome. We conclude that bedtime salivary cortisol measurement is a practical and accurate screening test for the diagnosis of Cushing's syndrome.

### Estrogen Receptors in Normal Endometrium and Endometriosis

*Hearns-Stokes; in collaboration with Zahn, Mayers, Segars*

We characterized the spatial and temporal localization of estrogen receptor (ER) alpha and beta in the endometrium and endometriosis explants of women with pelvic pain from endometriosis. Samples were obtained during laparoscopic laser excision surgery. Immunohistochemical staining for ER alpha showed that nuclear localization was greatest in the epithelium during the proliferative phase and decreased during the late secretory phase. ER beta showed a similar nuclear and epithelial cell preference, but the intensity of ER beta immunostaining was increased during the secretory phase. ER beta increased in endometriotic explants.

### Raloxifene as an Adjunctive Treatment for Endometriosis

*Stratton, Sinaii, Nahari, Zhu, Nilson, Lopez; in collaboration with Chow, Daly, Karp, Koziol, Wesley, Merino, Reynolds, Winkel, Azumi*

We also are conducting a randomized, double-blind, placebo-controlled study of surgery with or without raloxifene, Evista®, for the treatment of pain from endometriosis. To date, we have examined the utility of MRI in diagnosing endometriosis and correlated histopathology with laparoscopic findings. Our analysis suggests that MRI has limited diagnostic utility in a cohort of women with pelvic pain because it demonstrates a low detection rate of biopsy-proven endometriosis lesions and is relatively insensitive in determining whether a woman has endometriosis. In comparing surgical and histopathologic findings, only about 70 percent of endometriosis lesions seen at surgery have been demonstrated by biopsy.

### Autoimmune Disorders in Women with Endometriosis

*Sinaii, Stratton, Cleary, Ballweg*

In collaboration with the Endometriosis Association, we analyzed survey responses of almost 4,000 women with surgically diagnosed endometriosis. Almost all responders had pain (99 percent), and many reported infertility (41 percent). In this cohort of women with endometriosis, the rates of self-reported diagnoses of hypothyroidism, fibromyalgia, chronic fatigue syndrome, autoimmune diseases, allergies, and asthma were all significantly greater than the published prevalence in the general U.S. female population.

## SELECTED PUBLICATIONS

1. Doppman JL, Chrousos GP, Papanicolaou DA, Stratakis CA, Alexander HR, Nieman LK. Adrenocorticotropin-independent macronodular adrenal hyperplasia: an uncommon cause of primary adrenal hypercortisolism. Radiology. 2000;216:797-802.
2. Nieman LK. Diagnostic tests for Cushing's syndrome. Ann NY Acad Sci. 2002;970:112-118.
3. Papanicolaou DA, Mullen N, Kyrou I, Nieman LK. Nighttime salivary cortisol: a practical but assay-dependent test for the diagnosis of Cushing's syndrome. J Clin Endocrinol Metab. 2002;87:4515-4521.
4. Sinaii N, Cleary SD, Ballweg ML, Nieman LK, Stratton P. High rates of autoimmune and endocrine disorders, fibromyalgia, chronic fatigue syndrome and atopic diseases among women with endometriosis: a survey analysis. Hum Reprod. 2002;17:2715-2724.
5. Stratton P, Winkel CA, Sinaii N, Merino M, Zimmer C, Nieman LK. Location, color, size, depth, and volume may predict endometriosis in lesions resected at surgery. Fertil Steril. 2002;78:743-749.

6. Torpy DJ, Mullen N, Ilias I, Nieman LK. Association of hypertension and hypokalemia with Cushing's syndrome caused by ectopic ACTH secretion: a series of 58 cases. Ann NY Acad Sci. 2002;970:134-144.

## COLLABORATORS

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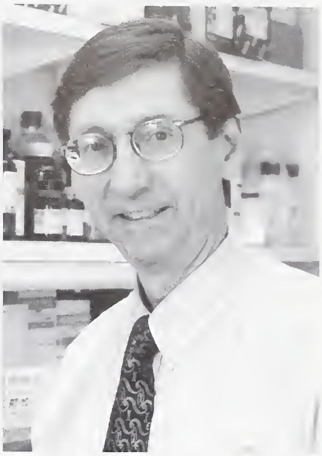
# REPRODUCTIVE ENDOCRINOLOGY AND INFERTILITY



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We investigate underlying causes and effectiveness of treatment for a variety of clinical reproductive disorders. The unit's studies are an integral facet of the clinical training program in Reproductive Endocrinology and Infertility. Our objective is to provide clinician-scientists with expertise in basic and clinical research related to reproduction.

### Uterine Fibroids

In the past year, we have begun collaboration with Dr. John Tsibris to investigate the causes of uterine fibroids. Uterine fibroids (also known as leiomyomata) are benign tumors of the womb that represent a common cause of reproductive disorders. Uterine fibroids afflict up to 22 percent of African American women and are a significant public health concern; surgeries for fibroids cost over $2 billion in the United States annually. Studies published this year suggested that fibroids might be distinguished from normal uterine tissue by abnormal expression of genes involved in growth and growth regulation. Based on these results, fibroids appear to be a feature of disordered differentiation of cells comprising the womb. In a collaboration with the Unit on Reproductive Medicine, led by Dr. Lynnette Nieman, we have initiated a clinical study to examine the medical treatment and molecular basis of fibroid disease and to expand on our initial findings.

### Mechanisms of Estrogen Action

Our basic research studies have continued to explore the relationship of the Brx protooncoprotein to estrogen-mediated responses. Findings this past year have implicated p38 mitogen-activated protein kinases in estrogen action and have further supported a signalling pathway involving cytoplasmic proteins in estrogen action. Current studies are directed to further elucidation of the physiologic role of the signalling pathway in reproduction.

## SELECTED PUBLICATIONS

1. Driggers PH, Segars JH, Rubino DM. The proto-oncoprotein Brx activates estrogen receptor by a p38 mitogen activated protein kinase pathway. J Biol Chem. 2001;276:46792-46797.
2. Frattarelli JL, Levi AJ, Miller BT. A novel method of determining ovarian size during in vitro fertilization cycles. J Assist Reprod Genet. 2002;19:39-41.
3. Segars JH, Driggers PH. Estrogen action and cytoplasmic signaling cascades. Part I: membrane associated complexes. Trends Endocrinol Metab. 2002;13:349-354.
4. Tsibris JCM, Segars J, Coppola D, Mane S, Wilbanks GD, O'Brien WF, Spellacy WN. Insights from gene arrays on the development and growth regulation of uterine leiomyomata. Fert Steril. 2002;78:114-121.

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# PATHOPHYSIOLOGY OF PREMATURE LABOR AND COMPLICATIONS OF PREMATURITY AND PRENATAL DIAGNOSIS OF CONGENITAL ANOMALIES

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Premature birth is the leading cause of perinatal mortality and morbidity worldwide. We have defined preterm labor as a syndrome and determined that at least 25 percent of all preterm births are to women with sub-clinical intrauterine infection. Moreover, we have provided evidence that a substantial number of premature neonates are critically ill before birth and proposed that the onset of premature labor has survival value in the context of intrauterine infection. This year, we conducted a number of studies focusing on the role of matrix-degrading enzymes in preterm premature rupture of membranes (PROM). After premature birth, congenital anomalies are the second leading cause of perinatal mortality in the United States. Advances in imaging techniques have allowed the detection of many anatomical defects before birth with ultrasound. The diagnostic power of three-dimensional ultrasound is illustrated by the improved detection of cleft lip, vasa previa, and congenital anomalies. This year, we reported the use of three-dimensional sonography in the prenatal diagnosis of micrognathia, absence of the nasal bone during the second and third trimester with trisomy 21, and the use of color power imaging to map fetal vascular anatomy.

## Role for Matrix Metalloproteinase in Spontaneous Rupture of the Fetal Membranes

*Chaiworapongsa, Edwin, Kim, Espinoza, Romero*

The fetal membranes normally rupture after the onset of spontaneous labor. PROM occurs in 10 percent of women at term and is associated with 30 to 40 percent of preterm deliveries. The mechanisms responsible for PROM remain poorly understood. Histologic studies of the site of rupture have demonstrated a zone of altered morphology characterized by thickening of the connective tissue components of the membranes and thinning of the cytotrophoblast layer and decidua as well as disruption of the interconnection between amnion and chorion. A significant decrease in the density of collagen type I, III, and V has been reported in the zone of altered morphology, which is in close proximity to the cervix. Increased intrauterine pressure during labor is thought to exert pressure over this area of the membranes, resulting in membrane rupture. Thus, the current hypothesis is that increased protease activity leads to an accelerated degradation of the extracellular matrix of the fetal membranes and that this degradation, coupled with biomechanical forces associated with labor, eventually leads to membrane rupture. In the context of intrauterine infection, proteases produced by bacteria or host cells could decrease membrane strength and elasticity. This year, we conducted studies on the role of matrix metalloproteinase 3 (MMP-3), also known as stromelysin 1, in preterm PROM and microbial invasion of the amniotic cavity; we also conducted studies on the role of neutrophil elastase and secretory leukocyte protease inhibitor. Importantly, we investigated whether the fetal carriage of single nucleotide polymorphisms for MMP-1 and MMP-9 is associated with preterm PROM.

### Neutrophil Elastase and MMP-3 in Preterm PROM

*Chaiworapongsa, Espinoza, Edwin, Romero*

Neutrophil elastase, a multifunctional serine protease stored in azurophilic granules of mature neutrophils, is capable of intracellular degradation of proteins during phagocytosis and extracellular degradation of connective tissue during an inflammatory process. Secretory leukocyte protease inhibitor (SLPI) is a natural neutrophil elastase inhibitor present in amniotic fluid, fetal membranes, and cervical mucus. An imbalance between neutrophil elastase and SLPI has been implicated as a mechanism of abnormal tissue destruction in chronic inflammatory diseases, and we propose that it may be involved in the mechanisms of membrane rupture. A series of studies indicated that preterm PROM was associated with a significant increase in the amniotic fluid concentration of neutrophil elastase and that microbial invasion of the amniotic cavity was associated with a significant increase in the amniotic fluid concentration of neutrophil elastase in women with preterm labor and intact membranes as well as in women with preterm PROM. Moreover, in the absence of microbial invasion of the amniotic cavity, preterm and term PROM were associated with a significant reduction in the amniotic fluid concentration of SLPI. These observations add strength to the concept that intra-amniotic inflammation and host proteases participate in the mechanism of membrane rupture.

Stromelysin 1 (MMP-3), produced in the context of infection, is able to activate the latent forms of other MMPs. In contrast to the observations that implicate neutrophil collagenase in the mechanism of membrane rupture, preterm PROM was not associated with an increase in MMP-3 concentrations in the amniotic fluid, although its concentrations increased in the context of intra-amniotic infection.

### Association of Genetic Predisposition to Preterm PROM with Single Nucleotide Polymorphisms for MMP-1 and MMP-9

*Romero*

Our previous studies have provided evidence for the participation of MMP-9 and MMP-1 in preterm PROM. Recent studies have reported functional polymorphisms for genes encoding these enzymes. We conducted two studies to determine if these polymorphisms would raise the expression of MMP-1 and MMP-9 by amnion cells and whether their frequency may be elevated in patients with preterm PROM. A polymorphism at -1607 in the MMP-1 promoter (an insertion of a guanine, G) creates a core Ets-binding site and increases promoter activity. The 2G promoter was more than twice as active as the 1G allele in amnion mesenchymal cells and a clone amnion cell line. Phorbol 12-myristate 13-acetate (PMA) increased mesenchymal cell nuclear protein binding with greater affinity to the 2G allele. Induction of MMP-1 mRNA by PMA was significantly greater in cells with a 1G/2G or 2G/2G genotype compared with cells homozygous for the 1G allele. When treated with PMA, the 1G/2G and 2G/2G cells produced greater amounts of MMP-1 protein than 1G/1G cells. We found a significant association between fetal carriage of a 2G allele and preterm PROM. Thus, the 2G allele has stronger promoter activity in amnion cells and confers increased responsiveness of amnion cells to stimuli that induce MMP-1; carriage of this polymorphism increases the risk of preterm PROM.

We studied the functional significance of a variable-number tandem repeat and a single nucleotide polymorphism (SNP) in the MMP-9 gene on promoter activity and their association with preterm PROM. The 14 CA-repeat allele was a stronger promoter than the 20 CA-repeat allele in amnion epithelial cells and WISH amnion–derived cells, but in THP-1 monocyte/macrophage cells the 14 and 20 CA-repeat alleles exhibited similar activities. An SNP at -1562 did not significantly affect promoter activity. A case-control study indicated that the 14 CA-repeat allele was more common in newborns delivered to mothers with preterm PROM than in those delivered at term. There was no association between the -1562 SNP and preterm PROM. Thus, the evidence suggests that cell host–dependent differences in MMP-9 promoter activity are related to the CA-repeat number and that fetal carriage of the 14 CA-repeat allele is also associated with preterm PROM.

### Three-Dimensional Ultrasonographic Presentation of Micrognathia

*Chaiworapongsa, Kalache, Romero*

Micrognathia refers to a facial malformation characterized by a small chin. The National Library of Medicine's Online Mendelian Inheritance in Man database includes 211 genetic conditions with such a malformation. Affected fetuses are at increased risk for an abnormal karyotype. An underdeveloped chin may be severe enough to cause polyhydramnios or respiratory obstruction after delivery. Micrognathia can complicate infant feeding and typically requires surgical repair. The prenatal diagnosis of micrognathia is difficult and largely subjective. We have used three-dimensional ultrasound to examine the fetal mandible and developed an approach for the diagnosis. We used orthogonal multiplanar views to obtain a midsagittal facial profile. The most important advantage of three-dimensional ultrasound is its ability to display a true midsagittal plane of the fetal face. Diagnostic inaccuracies, however, can result from inappropriate manipulation of multiplanar images and rendered volume data. One of the advantages of three-dimensional ultrasound surface rendering is that it enables referring physicians to counsel prenatal patients accurately.

### Evaluation of Spina Bifida by Three-Dimensional Ultrasonography

*Chaiworapongsa, Romero*

The most common and difficult-to-diagnose neural tube defect is spina bifida. Advances in treatment have greatly reduced mortality, but they have had minimal impact on long-term disability, which includes paraplegia, sensory deficits, spinal deformity, bowel dysfunction, and urinary incontinence. Prenatal ultrasonography can detect between 80 to 85 percent of fetuses with spina bifida. The location and extent of the lesion are critical factors in assessing the likelihood of long-term disability. By using two- and three-dimensional ultrasonography to determine the extent of vertebral defects among affected fetuses, we have developed a novel approach for the systematic examination of the fetal spine and the mapping of defects. We counted spinal levels independently from the most caudal thoracic vertebra with a rib (e.g., 12th thoracic rib) and manipulated a virtual cutting plane through a volume-rendered spine to generate optimal multiplanar views for this blinded analysis. We compared prenatal diagnosis with a postnatal analysis of bony spine defects derived from radiographic films or magnetic resonance imaging and used two-dimensional and three-dimensional ultrasonography to examine fetuses. For two-dimensional ultrasonography, the spinal level agreed to within one vertebral segment in six of nine infants. In contrast, three-dimensional ultrasonography agreed to within one vertebral segment in eight of nine infants. Three fetuses had vertebral defect levels on two-dimensional ultrasonography that were 1.5 to two segments away from postnatal findings. The same fetuses had results that were within one vertebral segment on three-dimensional ultrasonography. Volume rendering showed splayed vertebral pedicles and disrupted vertebrae. An intact meningeal sac was easily rendered in five of nine subjects. Multiplanar views were more informative than rendered views for localizing bony defects of the fetal spine.

### Nasal Bone Evaluation in Fetuses with Down Syndrome during the Second and Third Trimesters of Pregnancy

*Kalache, Chaiworapongsa, Romero*

The standard method for the prenatal diagnosis of trisomy 21 is karyotype of cells obtained through amniocentesis, a procedure with a 0.5 percent risk of pregnancy loss. In response to considerable interest in the identification of sonographic markers as an alternative diagnostic tool, recent studies suggest that a non-visualized nasal bone may identify fetuses with trisomy 21 in the first trimester of pregnancy. Yet, little information is available on the diagnostic potential of this sonographic finding in the second and third trimesters of pregnancy, which is when most sonograms are performed in the

United States. Three-dimensional ultrasound uses multiplanar views that allow for standardization of the plane for the examination of the fetal nasal bone. We conducted a study to determine the value of this technology in assessing the presence or absence of the fetal nasal bone in normal fetuses as well as in those with trisomy 21 and found that three-dimensional ultrasound allowed substantial agreement in scoring whether or not the nasal bone was visualized. Forty percent of fetuses with trisomy 21 had absence of the nasal bone. In contrast, we identified the nasal bone in 80 to 90 percent of normal fetuses. Our most important finding was that three-dimensional ultrasound allowed standardization of the diagnosis.

### Genetic Sonography, a Cost-Effective Method for Evaluating Women 35 Years and Older Who Decline Genetic Amniocentesis

*Romero*

Given that the frequency of aneuploidy increases with advancing maternal age, standard clinical practice calls for offering invasive testing (chorionic villus sampling or second-trimester amniocentesis) to women 35 years of age and older. Following genetic counseling, not all women elect invasive testing. Some patients inquire whether ultrasound examination of the fetus would provide additional information regarding their risk for aneuploidy or other genetic diseases. Accordingly, the sonographer or sonologist must be able to identify fetal ultrasound features associated with aneuploidy. Previous studies have indicated that the sensitivity of genetic ultrasound for detection of a fetus with trisomy 21 ranges from 25 to 91 percent and that the sensitivity for detecting all chromosomal abnormalities in women of advanced maternal age is 81 percent. In addition, an incremental cost is associated with genetic ultrasound. We conducted a study to determine if offering genetic ultrasound to patients who decline invasive testing is cost-effective and can increase the detection rate of trisomy 21. We determined the detection rate of trisomy 21, the number of pregnancy losses after amniocentesis, and the cost of detecting a single fetus with trisomy 21 in women 35 years and older. We concluded that offering genetic sonography followed by amniocentesis to patients 35 years and older who originally decline invasive testing for the diagnosis of trisomy 21 is cost-effective and results in a higher overall detection rate for trisomy 21 without an increased risk of pregnancy loss.

## SELECTED PUBLICATIONS

1. Chaiworapongsa T, Gervasi MT, Refuerzo J, Espinoza J, Yoshimatsu J, Berman S, Romero R. Maternal lymphocyte subpopulations (CD45RA+ and CD45RO+) in preeclampsia. Am J Obstet Gynecol. 2002;187:889-893.
2. Chaiworapongsa T, Romero R, Kim JC, Kim YM, Blackwell SC, Yoon BH, Gomez R. Evidence for fetal involvement in the pathologic process of clinical chorioamnionitis. Am J Obstet Gynecol. 2002;186:1178-1182.
3. Chaiworapongsa T, Romero R, Yoshimatsu J, Espinoza J, Kim YM, Park K, Kalache K, Edwin S, Bujold E, Gomez R. Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. J Matern Fetal Neonatal Med. 2002;12:19-27.
4. DeVore GR, Romero R. Genetic sonography: a cost-effective method for evaluating women 35 years and older who decline genetic amniocentesis. J Ultrasound Med. 2002; 21:5-13.
5. Ferrand PE, Parry S, Sammel M, Macones GA, Kuivaniemi H, Romero R, Strauss JF III. A polymorphism in the matrix metalloproteinase-9 promoter is associated with increased risk of preterm premature rupture of membranes in African Americans. Mol Hum Reprod. 2002;8:494-501.
6. Fujimoto T, Parry S, Urbanek M, Sammel M, Macones G, Kuivaniemi H, Romero R, Strauss JF. A single nucleotide polymorphism in the matrix metalloproteinase-1 (MMP-1) promoter influences amnion cell MMP-1 expression and risk for preterm premature rupture of the fetal membranes. J Biol Chem. 2002;277:6296-6302.
7. Helmig RB, Romero R, Espinoza J, Chaiworapongsa T, Bujold E, Gomez R, Ohlsson K, Uldbjerg N. Neutrophil elastase and secretory leukocyte protease inhibitor in prelabor rupture of membranes, parturition and intra-amniotic infection. J Matern Fetal Neonat Med. 2002;12:237-246.

8. Lee W. Chaiworapongsa T. Romero R. Williams R. McNie B. Johnson A. Treadwell M. Comstock CH. A diagnostic approach for the evaluation of spina bifida by three-dimensional ultrasonography. J Ultrasound Med. 2002; 21:619-626
9. Lee W. McNie B. Chaiworapongsa T. Conoscenti G. Kalache KD. Vettraino IM. Romero R. Comstock CH. Three-dimensional ultrasonographic presentation of micrognathia. J Ultrasound Med. 2002; 21:775-781.
10. Romero R. Espinoza J. Chaiworapongsa T. Kalache K. Infection and prematurity and the role of preventive strategies. Semin Neonatol. 2002;7:259-274.

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# ADVANCED TECHNOLOGIES FOR REHABILITATION RESEARCH

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We develop and apply advanced technologies that create new directions for basic, translational, and clinical rehabilitation research in support of the trans-rehabilitation research domain model, which postulates that our ability to understand disablement rests on linking knowledge of the impairment, functional limitation, and physical disability rehabilitation research domains. The scope of these advanced technologies encompasses a broad range that includes theoretical concepts, numerical models, computational methods, and instrumentation development. We also work to establish models of swallowing, speech, respiration, aerodigestive, and oral motor function across the life span in both normal individuals and individuals with various conditions, striving to understand normative functions that have an impact on clinical treatments and aid in the discovery of new strategies that may improve the lives of persons with swallowing, speech, and oral motor impairments. We are investigating the potential of real-time two- and three-dimensional ultrasound imaging to evaluate the prenatal development of the aerodigestive system so that we can identify fetal compromise early and prevent neonatal feeding and respiratory dysfunction. We are comparing the sensitivity and specificity of newer three-dimensional imaging to provide earlier detection of fetal anomalies, more accurate measures of growth, and greater resolution of fetal anatomy.

### Induced Acceleration Analysis for Rehabilitation

*Kepple, Siegel, Selbie, Stanhope*

By studying the contribution of net joint moments and individual muscle forces to the generation and control of human movement and the performance of activities of daily living, we hope to link a physically challenged person's capabilities to his or her ability to perform activities of daily living. Measuring these contributions is critical for defining adaptive movement control strategies and understanding the process whereby the strategy of movement control is selected and implemented.

Earlier efforts to determine the contribution of net muscular joint moments and individual muscle forces to human movement control in a rehabilitation context have been limited by the assumption that the relative contribution of muscular effort to the control of human movement is proportional to the magnitude of the effort and independent of the position of the body segments. A generalized coordinate formulation of the equations of motion shows that this assumption is incorrect and can lead to errors in interpreting human movement analysis data. We have extended existing gait analysis methodology by developing a generalized coordinate-based mechanical linkage model that can provide direct estimates of the influence of a given joint moment or muscle force on the movement of all joints, body segments, and overall task performance.

Our research revealed that the generation of upper body forward velocity during normal gait arises primarily from ankle joint plantar flexor push-off rather than through a controlled fall. We also found that the ankle joint plantar flexor muscle group produces forward acceleration of the upper body during periods in which the same muscle group acts eccentrically. The literature suggests that deceleration of the lower extremity swing limb is an additional source of forward velocity generation during gait. Our results indicate, however, that swing limb moments do not make a significant contribution to the forward acceleration of the upper body during normal walking. We examined the relative contribution of the lower extremity joint moments to the maintenance of upright posture (support) and found that, during the single limb support phase, ankle moments generate the greatest contribution to support. During double limb support, the knee and hip moments of the forward positioned leg increase their contributions to support.

We also examined the relative sensitivity of the joint accelerations to the joint moments during gait and found that, during the foot-flat phase, the ankle, knee, and hip joint accelerations are almost twice as sensitive to moments generated at the knee as to the moments at any other lower extremity joint. During the heel-off phase, the acceleration sensitivities at both the ankle and hip joints increase in response to moments at their own joint. We also found that some joint sensitivity directions change during late stance, indicating that the role played by a muscle group in controlling human motion can change entirely during a movement such as walking. It is clear from the data that there is significant redundancy in controlling the motion of the stance limb during gait. Our findings explain, in part, the ability of persons with muscle weakness to compensate via the use of alternative movement control strategies. We developed a method for estimating the source of mechanical energy exchange between segments and used it to determine the muscle groups responsible for the inter-segment transfer of mechanical energy during gait, showing that energy transfer depends on the sign of the joint moment rather than on the sign of the joint power and that pairs of net joint moments function in combination to balance energy flow through the leg and trunk segments during normal gait.

Future research plans call for developing a seamless integration of the induced acceleration techniques with the C-Motion Visual3D and Mechanical Dynamics ADAMS software and implementing a biomechanical model to determine the relative sensitivity of individual muscles for controlling motion during functional tasks of all lower extremity joints, sensitivities that are believed to be a measure of the compensatory capacity of a muscle to control the motion of all joints during human movement activities. We also intend to expand our analysis of joint moments to include the contributions of the individual muscle forces to the joint accelerations and to evaluate the utility of assistive technologies (e.g., instrumented walkers) to the control of human movement and performance of activities of daily living.

### Virtual Functional Anatomy

*Sheehan, Cohen, Rausch, Woodburn, Stanhope; in collaboration with Rebmann, Yao*

The Virtual Functional Anatomy (VFA) project is designed to fill the important gap in our knowledge of the relationship between normal or impaired joint structure/function and the functional movement limitations associated with performing activities of daily living. Our current focus is to develop and ultimately validate a combined set of tools that will enable the accurate and precise measurement, analysis, and visualization of three-dimensional (3-D) static and dynamic musculoskeletal anatomy (i.e., bone shape, skeletal kinematics, tendon and ligament strain, muscle force, and joint space). We plan to combine MRI imaging and analysis capabilities with the a highly accurate, imaging-based measurement and analysis technique for the non-invasive quantification of complete joint anatomy and tissue dynamics during functional movements. This will require the development of a method for creating 3D digital images of loaded and moving joint tissues (bone, cartilage, and connective tissues) to reveal joint contact patterns and tissue loads. We will also evaluate the variability of bone shape and the sensitivity of defined joint posture (translation and rotation of one bone relative to another) to osteo-based coordinate system definition. We intend to use these capabilities to document and evaluate the function of normal and impaired joint structures (e.g., ACL rupture and patellar tracking syndrome) under simulated conditions experienced during activities of daily living.

We have demonstrated the feasibility of studying musculoskeletal kinematics with Cine–phase constrast (PC) and fast-PC MRI (the primary component of VFA) and determined the accuracy of Cine-PC MRI and, by extension, fast-PC MRI in the measurement of skeletal kinematics. We have determined the precision of Cine-PC and fast-PC MRI in the measurement of skeletal kinematics and measured knee joint kinematics and patellar tendon strain during a leg extension task, under load. Work is ongoing to define static and dynamic measures of patellar tracking abnormalities as a potential source of patello-femoral (PF) pain and to determine which three-dimensional imaging sequence is the most accurate and precise in defining skeletal landmarks, cartilage thickness, and joint space profiles. We are developing a technique to define coordinate systems based on skeletal landmarks and determining their sensitivity to small variations in landmark definition.

### Ultrasonic Evaluation of the Development of the Fetal Upper Aerodigestive Tract

*Sonies, Miller; in collaboration with Macedonia, Merke*

We investigate the prenatal development of the upper aerodigestive tract, postulating that intrauterine behaviors of the fetus may provide guidance for appropriate postnatal feeding interventions. Our investigation of fetal swallowing has identified potential limitations in the use of color Doppler (CD) imaging for the detection of amniotic fluid flow and discrimination of respiratory from ingestive activity. Whereas CD imaging represents the spectrum of flow velocity and direction, power Dopper (PD) imaging represents the amplitude, or intensity, of the Doppler signals. Thus, PD imaging offers increased sensitivity for detection of low-amplitude signals regardless of direction. Because PD imaging is less angle-dependent, amniotic fluid flow during ingestion or respiratory activity can be detected across a broad range of fetal postures that previously restricted data collection. Therefore, we investigated the potential of real-time two- and three-dimensional ultrasound imaging to evaluate prenatal development of the aerodigestive system as a means for early identification of fetal compromise and prevention of neonatal feeding and respiratory dysfunction. Sensitivity and specificity of newer three-dimensional imaging provide earlier detection of fetal anomalies, more accurate measures of growth, and greater resolution of the fetal anatomy. We identified the course of postnatal development (four and eight months of age) of feeding swallowing skills in all newborns with upper aerodigestive system difficulties.

The literature has yet to define satisfactorily the clinical indicators of maturational “readiness” for feeding of the preterm or medically fragile neonate. The initiation of oral feeding of neonates is held to be at approximately 34 weeks gestational age (GA), when it is presumed that ingestive skills are mature enough for successful feeding. We studied two fetal-maternal populations: normal healthy controls (18 to 28 weeks GA) and the “at-risk” fetus (e.g., cardiomyopathy, *in vitro* fertilization, intrauterine growth retardation, substance abuse). We established anthropomorphic measures of the upper aerodigestive system and profiles of fetal activity aerodigestive development and feeding and identified fetal and maternal prenatal risk factors predictive of neonatal feeding deficits. We have preliminary evidence that proficient neonatal suckling movements, representing an extensive integration of intrauterine upper aerodigestive skills, emerge before 34 weeks GA. In a range of abnormal cases, prenatal identification of differences in ingestive function may be useful in determining the degree of postnatal feeding skills. We have also observed that a significant linear increase in pharyngeal and lingual growth occurs across gestation in healthy subjects (similar linear patterns are not found for laryngeal and facial structures) and that significant differences in pharyngeal growth patterns occur between normal and at-risk cases. Our analysis of behavioral patterns reveals the developmental progression of lingual movements similar to postnatal suckling patterns that advance from simple anterior thrusts to more complex bolus cupping and anterior-posterior motion. We also found that the pharyngeal–laryngeal responses demonstrate a range of periodic–aperiodic, complete–incomplete activity patterns discernible as respiratory-ingestive motor activity, that fetal swallowing demonstrates unique patterns of movement occurring after fetal stimulatory behavior, and that the complex integrated movement patterns of the mature fetal swallow emerge before 34 weeks GA.

## SELECTED PUBLICATIONS

1. Macedonia C, Miller JL, Sonies BC. Power Doppler imaging of the fetal upper aerodigestive tract: a four-point standardized evaluation. J Ultrasound Med. 2002;21:869-878.
2. Manal K, McClay I, Richards J, Galinat B, Stanhope S. Knee moment profiles during walking: errors due to soft tissue movement of the shank and the influence of the reference coordinate system. Gait Posture. 2002;15:10-17.
3. Sheehan FT, Drace JE. Human patellar tendon strain (a non-invasive, in vivo study). Clin Orthop. 2000;370:201-207.
4. Sheehan FT, Pappas G, Drace JE. In vivo measures of musculoskeletal dynamics using cine phase contrast magnetic resonance imaging. In: Herzog W, ed. Skeletal muscle mechanics: from mechanism to function. Hoboken NJ: John Wiley & Sons, 2000;343-363.
5. Sonies BC, Chi-Fishman G, Miller JL. Ultrasound imaging and swallowing. In: Jones B, ed. Normal and abnormal swallowing: imaging in diagnosis and therapy. 2nd edition, New York: Springer, 2002;in press.

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# STUDIES ON DNA REPLICATION, REPAIR AND MUTAGENESIS IN EUKARYOTIC AND PROKARYOTIC CELLS

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Under optimal conditions, the fidelity of DNA replication is extremely high, with roughly one error occurring for every $10^{10}$ bases replicated. However, DNA is subjected to endogenous and exogenous DNA-damaging agents *in vivo*. Even though all organisms have evolved elaborate repair pathways to deal with damaging agents, the repair pathways rarely operate with 100 percent efficiency and, as a consequence, the persisting DNA lesions are replicated, but with much lower fidelity than undamaged DNA. The replication process is commonly referred to as translesion DNA synthesis (TLS) or translesion replication (TR) and is often facilitated by one or more of the "Y-family" of DNA polymerases, which are phylogenetically conserved from bacteria to humans. Therefore, our general aim is to understand the molecular mechanisms by which mutations are introduced into damaged DNA. In the past, most of our efforts have focused on *Escherichia coli* primarily because its cellular, molecular, and genetic characterization was the most advanced of any organism. However, the past few years have witnessed tremendous progress in understanding the mutation process in higher organisms, including *Saccharomyces cerevisiae* and human cells. As a consequence, we have investigated the process in all three kingdoms of life: bacteria, archaea, and eukaryotic cells.

## Translesion Replication in *Escherichia coli*

*Woodgate; in collaboration with Goodman, Lawrence, Jerina*

In *E. coli*, efficient translesion replication of many DNA lesions occurs only when the UmuC protein interacts with a dimer of UmuD' to form a heterotrimeric complex of $\text{UmuD'}_2\text{C}$ (known as *E. coli* pol V). Because pol V is a low-fidelity enzyme, its activities within the cell are strictly controlled at the transcriptional level as well as at multiple post-translational steps. Biochemical experiments performed in collaboration with Myron Goodman demonstrated, for example, that the *in vitro* catalytic activity of pol V is greatly stimulated through a physical interaction with the RecA protein. Interestingly, these studies suggest that two distinct biochemical modes of RecA binding are necessary for pol V–catalyzed translesion replication. One RecA mode is characterized by a strong stimulation in nucleotide incorporation either directly opposite a lesion or at undamaged template sites, though by the absence of lesion bypass. A separate RecA mode is necessary for translesion synthesis. In the past, it was believed that RecA's stimulatory effect on pol V required the formation of a RecA nucleoprotein filament on single-stranded DNA. We tested this model by using lesion-containing templates in which the lesion was located in a short gap or at the end of a DNA template where no RecA nucleoprotein filament can form, and we discovered that there was nevertheless efficient lesion bypass. Based on our *in vitro* observations, we hypothesized that translesion replication therefore occurs when pol V interacts with two RecA molecules, first at a 3'-primer end upstream of a template lesion, where it stimulates pol V's catalytic activity, and, subsequently, at a site immediately downstream of the lesion, where RecA co-catalyzes lesion bypass. Our data also suggest that, while assembly of a RecA nucleoprotein filament may be required to target pol V principally to a site of DNA damage and to stabilize pol V-RecA interactions at the lesion *in vivo*, formation of a nucleoprotein filament *per se* is not an absolute requirement for translesion replication.

In addition to pol V, *E. coli* possesses four other DNA polymerases. Like pol V, pol II and pol IV are induced in response to cellular DNA damage, suggesting that they may play a role in cell survival and possibly in translesion replication. In a recent collaborative study with Myron Goodman and Donald Jerina, we investigated the ability of each of these polymerases to facilitate *in vitro* lesion bypass of benzo[*a*]pyrene diol epoxide (BaP DE), a common carcinogen found in cigarette smoke. In general, pol V bypassed deoxyadenosine BaP DE adducts with moderate accuracy and considerably higher efficiency than pol II or pol IV. In contrast, however, we observed very little pol V–dependent replication past the deoxyguanosine BaP DE adduct. Remarkably, we found that pol IV in fact bypasses deoxyguanosine BaP DE adducts with the highest efficiency and fidelity. Compared with the isogenic wild-type *E. coli* strain, deoxyguanosine BaP DE–adducted M13 DNA yielded four-fold fewer plaques *in vivo* when transfected into damage-induced Δ*dinB* (pol IV) mutant cells, a finding consistent with the *in vitro* data showing that pol IV is by far the best at copying the deoxyguanosine BaP DE adduct.

In a related study (in collaboration with Christopher Lawrence), we investigated whether one (or more) of *E. coli*'s five DNA polymerases might be able to substitute for pol V in the bypass of a *cys-syn* thymine–thymine cyclobutane dimer (a common DNA adduct generated upon exposure to sunlight). Indeed, although little replication past a T–T dimer normally takes place in *E. coli* in the absence of pol V, we previously observed as much as half the wild-type bypass frequency in pol V–deficient (Δ*umuDC*) strains when the 3' to 5' exonuclease proofreading activity of the pol III ε subunit was also disabled (by *mutD5,* a dominant negative mutation in *dnaQ*). We investigated this phenomenon by examining dimer bypass frequencies in Δ*umuDC* (polV$^-$) *mutD5* (proofreading-deficient*)* strains that were also deficient for pol I, pol II, and pol IV, both singly and in all combinations. Surprisingly, dimer bypass frequencies did not decrease in any of these strains; indeed, some increased to levels approaching those found in strains containing pol V. Efficient dimer bypass was, however, entirely dependent on the proofreading deficiency imparted by *mutD5*, indicating that bypass was probably performed by the *mutD5* pol III enzyme itself. Collectively, our prokaryotic lesion-bypass studies support the hypothesis that *E. coli* pols III, IV, and V may all play roles, to varying extents, in copying template DNA lesions. The extent to which they are ultimately utilized appears to depend on the particular lesion encountered and the cellular abundance/availability of each polymerase within the cell.

### Replication Restart in *Escherichia coli*

*Woodgate; in collaboration with Goodman*

In general, translesion replication appears to be used as a "last resort," presumably as it is inherently error-prone and leads to an increase in cellular mutagenesis. Instead, by means of an error-free "repair" process called replication restart, UV-irradiated *E. coli* rapidly resumes DNA synthesis after a transient inhibition. To elucidate the role of several key proteins involved in this process, we analyzed the time dependence of replication restart in strains carrying a combination of mutations in *lexA*, *recA*, *polB* (pol II*)*, *umuDC* (pol V*)*, *priA*, *dnaC*, *recF*, *recO*, or *recR*. We observed that both pol II and the origin-independent primosome-assembling function of PriA are essential for the immediate recovery of DNA synthesis after UV-irradiation. In their absence, translesion replication eventually occurs about 50 minutes post-UV and is pol V–dependent. In a wild-type, *lexA*$^+$ background, mutations in *recF*, *recO*, or *recR* block both pathways. We obtained similar results with a *lexA*(Def) *recF* strain. However, *lexA*(Def) *recO* or *lexA*(Def) *recR* strains, while unable to facilitate PriA-pol II–dependent restart, were able to perform pol V–dependent translesion replication. The defects in restart attributed to mutations in *recF*, *recO*, or *recR* were suppressed in a *recA730 lexA*(Def) strain expressing constitutively activated RecA (RecA*). Our data suggest, therefore, that in a wild-type background, RecF, O, and R are important for the induction of the SOS response and the formation of RecA*-dependent recombination intermediates necessary for PriA/Pol II-dependent replication restart. In contrast, only RecF is required for the activation of RecA that leads to the formation of pol V (UmuD'$_2$C) and facilitates translesion replication.

### Y-Family Polymerases in Archaea

*Boudsocq, Valjavec-Gratian, Woodgate; in collaboration with Yang, Kunkel*

We recently identified and cloned a Y-family DNA polymerase from the thermostable archaeon *Sulfolobus solfataricus* P2, which we called DNA polymerase IV (Dpo4). In a collaborative study with Wei Yang, we used x-ray diffraction to solve the crystal structure of Dpo4 in a ternary complex with an undamaged template DNA and an incoming dexoyribose nucleoside triphosphate. The studies revealed that, like all DNA polymerases, Dpo4 possesses a topology similar to a right hand with protein domains that resemble "fingers," a "palm," and a "thumb." Dpo4 also possesses a unique domain called the "little finger" that helps the enzyme bind to DNA. Interestingly, the domains are all small while the active site of the enzyme is large and solvent-exposed.

Phylogenetic analysis reveals that the Dpo4 protein falls within the DinB branch of the Y-family of DNA polymerases. The best examples of this branch of the Y-family polymerases are *E. coli* pol IV and human polκ, which are both known to make -1 frameshift mutations at high frequency. We were therefore interested in determining if the same might be true of Dpo4. In a collaborative study with Thomas Kunkel, we did indeed find Dpo4 to have a low frameshift fidelity in an *in vitro* gap-filling assay. It frequently generates -1 base pair deletions at non-iterated nucleotides and especially at cytosines flanked by a 5'-template guanine. We also found that Dpo4 generates all 12 base–base mismatches at high rates, 11 of which are similar to those of its human homolog, polκ. Surprisingly, Dpo4 generated C•dCMP mismatches at an unusually high average rate and preferentially at a cytosine flanked by 5'-template guanine. In more recent structural studies, we have been able to co-crystallize Dpo4 as it facilitates translesion replication of a synthetic abasic site. We find that the structurally rigid catalytic core of the polymerase binds to an incoming nucleotide regardless of whether it pairs with the correctly aligned template base. The limited protein–DNA interactions in the active site of the enzyme allow for rearrangements of the template–primer, including primer–template misalignment and flipping of bases into the minor groove, so as to avoid the abasic site and achieve base pairing with the incoming nucleotide.

Taken together, our *in vitro* assays and structural analysis of Dpo4-dependent lesion bypass suggest a molecular mechanism for Dpo4 replication. In this model, a nucleoside triphosphate is incorporated into the active site of the enzyme, and the primer and/or template DNA is manipulated until a correct base-pair is formed. Subsequent realignment of the primer-terminus within the active site of the polymerase therefore gives the appearance of a disproportionately high frequency of mispair formation (as is the case with C–C mispairs where the 5' template base is guanine). If the primer/terminus does not realign, it gives rise to a –1/+1 frameshift event, as observed in the case of certain DNA lesions, such as the abasic site.

### Human DNA Polymerase ι–Dependent Lesion Bypass

*Chumakov, Vaisman, Woodgate; in collaboration with Jerina, Hanaoka*

Another polymerase that has been extensively characterized is human DNA polymerase ι (polι), which we identified about two years ago. Pι is a Y-family DNA polymerase and a paralog of polη. A characteristic of polη is its ability to bypass thymine–thymine dimers accurately and efficiently both *in vitro* and *in vivo*. Indeed, humans with defects in polη exhibit the

*Xeroderma Pigmentosum Variant* phenotype, a disease in which afflicted individuals are extremely sensitive to sunlight, are cancer-prone, and often die at a young age unless exposure to sunlight is kept to a minimum. It therefore seems reasonable to expect that similar defects in the related polι enzyme might also lead to a human disease with a DNA repair–associated defect.

Given the phylogenetic relationship between polι and the highly efficient and accurate lesion-bypassing enzyme, pol η, we wished to determine the *in vitro* lesion-bypassing properties of polι. Our collaborators Fumio Hanaoka and Donald Jerina kindly provided us with a variety of lesion-containing oligonucleotide templates that we used in *in vitro* replication assays. We discovered that polι is unlike polη in that it bypasses very few lesions efficiently and, when it does insert a base(s) opposite the lesion, it is highly erroneous. Even in cases where human polι can efficiently insert a base opposite a lesion (such as a synthetic abasic site, the 3'T of a 6–4 thymine–thymine pyrimidine–pyrimidone photoproduct or opposite benzo[*a*]pyrene diol epoxide or benzo[*c*]phenanthrene diol epoxide deoxyadenosine adducts), further extension is often limited. Our *in vitro* studies showed that, while polι was unable to extend the correctly inserted dTMP opposite a benzo[*a*]pyrene diol epoxide deoxyadenosine lesion, it could be extended by another human Y-family DNA polymerase, polκ, so as to achieve complete lesion bypass. Based on our *in vitro* observations, we therefore hypothesize that polι and polκ act together to facilitate the error-free bypass of diol epoxide–adducted deoxyadenosine *in vivo* and, in doing so, protect humans from the carcinogenic effects of exposure to benzo[*a*]pyrene diol epoxides.

### *In Vivo* Characterization of DNA Polymerase ι

*McLenigan, McDonald, Plosky, Vidal, Woodgate; in collaboration with Lehmann*

Despite much progress in recent years in understanding the molecular mechanisms of low-fidelity DNA synthesis on undamaged DNA templates as well as translesion replication *in vitro*, little is known about how the Y-family polymerases, and polι in particular, are regulated *in vivo*. In a collaborative study with Alan Lehmann, we constructed recombinant fluorescent-tagged human polι and examined the subcellular localization of polι within a living cell. The studies revealed that, even though polι lacks an obvious nuclear localization signal, it is predominantly localized in the nucleus, where it associates with the cell's normal replication machinery. Following DNA damage, polι accumulates into discrete foci at sites of stalled replication forks. Interestingly, the pattern of foci formation is identical to that previously reported by Lehmann and colleagues for the related Y-family polymerase, polη, suggesting that damage-induced polι- and polη-foci formation is tightly coordinated within the cell. We discovered that polη and polι interact with each other physically and that the C-terminal 224 amino acids of polι are sufficient for both the interaction with polη and its relocalization into replication foci. Our data suggest, therefore, that human polη and polι may coexist in a larger holoenzyme complex whereby their lesion-bypassing activities can be coordinated in response to DNA damage and that both enzymes may play a general role in maintaining genomic integrity as well as in participating in translesion replication.

## SELECTED PUBLICATIONS

1. Borden A, O'Grady PI, Vandewiele D, Fernández de Henestrosa AR, Lawrence CW, Woodgate R. *Escherichia coli* DNA polymerase III can replicate efficiently past a T-T *cis-syn* dimer if DNA polymerase V and the 3' to 5' exonuclease proofreading function encoded by *dnaQ* are inactivated. J Bacteriol. 2002;184:2674-2681.
2. Boudsocq F, Ling H, Yang W, Woodgate R. Structure-based interpretation of missense mutations in Y-family DNA polymerases and their implications for polymerase function and lesion bypass. DNA Repair. 2002;1:343-358.
3. Frank EG, Sayer JM, Kroth H, Ohashi E, Ohmori H, Jerina DM, Woodgate R. Translesion replication of benzo[*a*]pyrene and benzo[*c*]phenanthrene diolexpoxide adducts of deoxyadenosine and deoxyguanosine by human DNA polymerase ι. Nucleic Acids Res. 2002;30:5284-5292.

4. Gonzalez M, Woodgate R. The "tale" of UmuD and its role in SOS mutagenesis. BioEssays. 2002;24:141-148.
5. Kannouche P, Fernández de Henestrosa AR, Coull B, Vidal A, Gray C, Zicha D, Woodgate R, Lehmann AR. Localisation of DNA polymerases η and ι to the replication machinery is tightly co-ordinated in human cells. EMBO J. 2002;21:6246-6256.
6. Kokoska RJ, Bebenek K, Boudsocq F, Woodgate R, Kunkel TA. Low fidelity DNA synthesis by a Y family DNA polymerase due to misalignment in the active site. J Biol Chem. 2002;277:19633-19638.
7. Pham P, Seitz EM, Saleliev S, Shen X, Woodgate R, Cox MM, Goodman MF. Two distinct modes of RecA action are required for DNA polymerase V-catalyzed translesion synthesis. Proc Natl Acad Sci USA. 2002;99:11061-11066.
8. Rangarajan S, Woodgate R, Goodman MF. Replication restart in UV-irradiated *Escherichia coli* involving pols II, III, V, PriA, RecA and RecFOR proteins. Mol Microbiol. 2002;43:617-628.
10. Shen X, Sayer JM, Kroth H, Pönten I, O'Donnell M, Woodgate R, Jerina DM, Goodman MF. Efficiency and accuracy of SOS-induced DNA polymerases replicating benzo[*a*]pyrene-7,8-diol 9,10-epoxide A and G adducts. J Biol Chem. 2002;277:5265-5274.
11. Vaisman A, Frank EG, McDonald JP, Tissier A, Woodgate R. Polι-dependent lesion bypass *in vitro*. Mutat Res. 2002;510:9-22.
12. Vandewiele D, Fernández de Henestrosa AR, Timms AR, Bridges BA, Woodgate R. Sequence analysis and phenotypes of five temperature sensitive mutator alleles of *dnaE*, encoding modified alpha-catalytic subunits of *Escherichia coli* DNA polymerase III holoenzyme. Mutat Res. 2002;499:85-95.

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# PHYSICAL PRINCIPLES OF BIOMOLECULAR RECOGNITION, SELF-ASSEMBLY, AND REGULATION

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Interactions between various biological macromolecules control protein folding and assembly, DNA packing, protein-DNA interactions, tissue formation and stability, and many other processes. We study recognition reactions between biological macromolecules with a particular emphasis on the pathology of collagen fiber formation in brittle bone disease (osteogenesis imperfecta or OI) and other disorders. Over the years, we have found that collagen interactions and fiber formation share a number of physical principles with interactions involving DNA and other biological helices. For instance, we discovered that the helical symmetry of a molecular backbone determines not only the pattern of spots on x-ray images but also sequence homology recognition, condensation, and mesomorphism of DNA as well as temperature-favored assembly and structure and stability of collagen fibers. Our main goal is to achieve a better understanding of these fundamental principles and to use the gained knowledge for diagnostics, characterization, and treatment of OI and other diseases. In collaboration with NICHD and extramural clinicians and scientists, we strive to bring our expertise in physical biochemistry and theory to molecular and cell biology and to clinical research and practice.

## Thermal Stability, Metabolism, Function, and Pathology of Type I Collagen

*Kuznetsova, Makareeva, Mertz, Leikin*

Type I collagen is the most abundant animal protein; it forms the matrix of bone, skin, and other tissues. It was taken for granted that type I collagen triple helix melts several degrees *above* body temperature in all species from Arctic fish to humans. However, the collagen helix is less stable than expected. We demonstrated that monomeric collagen slowly melts at several degrees *below* body temperature. Its equilibrium state at body temperature is a random coil rather than a triple helix. Apparently, cells use molecular chaperones to fold it. Once secreted from cells and processed by enzymes, collagen helices begin to unfold. We found that micro-unfolding of the least stable domains triggers self-assembly of fibers where helices are protected from complete unfolding. We are now beginning to look more closely at the molecular details of micro-unfolding and at mechanisms relating changes in collagen thermal stability to connective tissue pathology caused by mutations.

## Thermal Stability of Type I Collagen from OI Patients with Glycine Substitutions and Deletions/Insertions in the Triple Helix

*Kuznetsova, Makareeva, Mertz, Leikin; in collaboration with Cabral, Marini*

The most common causes of moderate to lethal OI are glycine substitutions and deletions or insertions of small fragments in the $(\text{Gly-X-Y})_n$ sequence of collagen triple helix. Many researchers reported changes in the thermal stability of collagen accompanying these mutations. However, thermal stability of mutant collagens was previously studied exclusively by the chymotrypsin/trypsin digestion assay. By comparing a variety of techniques and undertaking careful analysis of denaturation kinetics, we demonstrated that, while the chymotrypsin/trypsin assay yields useful information on rapid collagen micro-unfolding, it cannot be used for reliable evaluation of changes in the overall thermal stability of the protein. Therefore we began a systematic analysis of the thermal stability of mutant collagens from the NIH collection of OI patients. Our study has already produced a number of surprising results. First, we found that Gly-Ser and Gly-Cys substitutions in different locations on the triple helix produce substantially different effects on the melting temperature of the protein. It appears that the position of the mutation within certain domains is more important than the identity of the substituting residue or its immediate local environment. Second, mutations at the N-terminal end of the molecule appear to cause particularly strong changes in thermal stability as well as distinct effects on the melting temperature of procollagen and collagen, which is not the case for mutations located in the middle of the triple helix. Third, for mutations causing substantial changes in the melting temperature, differential scanning calorimetry achieves, with 1 to 2 percent accuracy, deconvolution of melting peaks and determination of the fraction of mutant chains in different species, allowing comparison of the fraction of mutant chains at the level of secreted protein. Our experience with other proteins suggests that the fraction of secreted mutant chains may differ substantially from that predicted by expression analysis at the mRNA level. It was generally accepted that understanding the genotype-phenotype relationship is difficult without knowing this fraction, but no techniques for its accurate measurement (except for Cys mutations) had previously been developed.

### Changes in Thermal Stability and Micro-Unfolding Pattern of Collagen Helix Resulting from the Loss of α2(I) Chain

*Kuznetsova, Leikin; in collaboration with McBride*

Spontaneous mutations producing a completely nonfunctional α2 chain of type I collagen have been described in humans and mice, revealing that $\alpha1(\text{I})_3$ homotrimers replace type I molecules. The latter appear to be functional, but homozygous mutants exhibit severe OI. Previous *in vitro* studies delineated several subtle changes in thermal stability, fibrillogenesis, and intermolecular interactions between hetero-and homotrimers, but the exact role of the α2 chain in these processes still remained largely unclear. Some of the most puzzling findings concerned a substantially different temperature dependence of fibrillogenesis and intermolecular forces, suggesting that the role of the α2 chain in thermal stability of type I collagen may be even more pronounced than previously thought. We revisited this question in more detail by using purified tendon collagen from wild-type ($\alpha1(\text{I})_2\alpha2(\text{I})$ heterotrimers) and *oim* ($\alpha1(\text{I})_3$) mice as well as artificial $\alpha1(\text{I})_3$ homotrimers obtained by refolding of rat tail tendon collagen. In contrast to earlier reports, we established that *oim* homotrimers melt at about 2°C higher than wild-type heterotrimers, as measured by differential scanning microcalorimetry. We also found that the former melt about 100 times more slowly than the latter as measured by isothermal circular dichroism. Detailed analysis of micro-unfolding kinetics at different temperatures revealed the following: initial micro-unfolding of *oim* homotrimers and wild-type heterotrimers occurs at approximately the same rate, but at different sites; the weakest spot on the *oim* triple helix appears to be about 100 amino acids from the C-terminal end within the cyanogen bromide peptide CB6, consistent with the previous finding; the same micro-unfolding site is, however, also present in wild-type collagen, although the weakest spot of the latter is located close to the N-terminal end of CB8; and the overall patterns of micro-unfolding sites in type I hetero- and homotrimers are substantially different. We found virtually no post-translational over-modification of *oim* mouse tendon collagen. Moreover, thermal stability and micro-unfolding of artificial rat tail tendon homotrimers were similar to *oim* homotrimers. Thus, the observed changes are associated with the difference in the amino acid composition of the α1(I) and α2(I) chains rather than with the post-translational modification.

### Physical Biochemistry of G349C Collagen

*Kuznetsova, Leikin; in collaboration with Forlino, Cabral, Marini*

The first knock-in murine model for OI, the Brtl mouse, was recently described. A classical glycine substitution was introduced into one COL1A1 gene, resulting in α1(I) G349C chains. The Brtl phenotype exhibits autosomal dominant transmission. It resembles type IV human OI with a wide range of severity, from moderate to lethal. Last year, we extended structural and functional studies of collagen from these mice. We observed approximately 25 percent mutant α1(I) chain dimers in collagen extracts from tendon, skin, and bone in all heterozygous animals, regardless of age and disease severity, in molecules secreted from fibroblast and osteoblast cell cultures, and in saline, acetic acid, and pepsin extracts from extracellular matrix. Thus, mutant molecules are just as well incorporated into the matrix as normal collagen helices, forming mature covalent cross-links with the same efficiency. By using amino acid analysis and differential gel electrophoresis, we detected only minimal post-translational over-modification of mutant molecules in cell cultures and in animal tissues, and its extent was similar in lethal and non-lethal animals but varied between different tissues. We found no changes in the thermal stability and rate of thermal denaturation of mutant collagen, except for a slight broadening of denaturation peaks in differential scanning microcalorimetry. The broadening is apparently related to the heterogeneity of the protein population, which consists of a mix of molecules with no, one, and two mutant chains. The extent of heterogeneity (melting peak width) correlated with the size of fibrils formed upon *in vitro* fibrillogenesis. Measurements of intermolecular forces in tendons and demineralized bones revealed no changes in collagen-collagen interactions. Unlike many other reported Gly→Cys mutations, this substitution appears to be remarkably well compensated. It causes no kink or chain register shift in the triple helix and no changes in helix stability or interactions. The only substantial molecular pathology we were able to detect by *in vitro* studies of mouse tissues was a disruption of quasi-crystalline lateral packing of helices in tendons, which appears to be associated with abnormal collagen interaction with other matrix components, most likely glycosaminoglycans. Abnormal collagen interactions with glycosaminoglycans and proteoglycans may cause fibril disorganization in bone observed in some of the animals and result in bone fragility. Verification of this hypothesis is the focus of our present studies.

### Register Shift, Abnormal Extracellular Processing, and Abnormal Matrix Incorporation of Type I Collagen with Triplet Duplication Mutation in Lethal OI

*Makareeva, Kuznetsova, Leikin; in collaboration with Cabral, Colige, Marini*

The majority of collagen mutations causing OI are glycine substitutions that disrupt formation of the triple helix. A rare type of collagen mutation consists of a duplication or deletion of one or two Gly-X-Y triplets. The mutation shifts the register of collagen chains with respect to one another in the helix but does not interrupt the triplet sequence, and yet it causes severe clinical phenotypes. In this study, we investigated the biochemical consequences of the realignment of X and Y position interactions along the helix resulting from Gly-Ala-Hyp triplet duplication at AA 868-876. We previously reported a decrease in the melting temperature of about 2°C for helices with one mutant chain and about 6°C for helices with two mutant chains.

We recently developed a novel assay allowing us to detect enzymatic co-processing of mutant and wild-type collagens in the same reaction mixture by differential fluorescent labeling of the species. *In vitro* N-proteinase cleavage demonstrated that binding of the enzyme was not affected. Nevertheless, we found that N-propeptide cleavage is slower in collagen with the triplet duplication than in controls or procollagen with an α1(I) G832S substitution, showing that the register shift persists through the entire helix. The register shift causes substantially faster cleavage of mutant protein by mammalian collagenase. The long collagenase cleavage fragment (which does not contain the mutation site but has an altered chain register due to mutation) is more thermally stable than a similar fragment from control protein or protein with an α1(I) G832S substitution. Apparently the decrease in the overall melting temperature is associated with structural changes that occur in the C-terminal end of the molecule beyond the collagenase cleavage site. We also found abnormal cleavage by chymotrypsin and trypsin at sub-melting temperatures, indicating an altered local micro-unfolding pattern in the mutant protein consistent with a substantial reduction in the thermal stability of the C-terminal end of the molecule.

The register shift affects not only the protein thermal stability and extracellular processing but also disrupts incorporation of mutant collagen into fibrils and matrix. Proband fibrils formed inefficiently *in vitro,* with a four-fold higher concentration required for the same fibrillogenesis rate and a similar amount of formed fibers. Only 30 percent of proband monomers incorporated into fibrils. The fibrils that did form contained only normal helices and helices with a single mutant chain. Helices with two mutant chains and a significant portion of helices with one mutant chain did not form fibrils. In matrix deposited by proband fibroblasts, mutant chains were abundant in the immaturely cross-linked fraction but constituted a minor fraction of maturely cross-linked chains. Since double mutant molecules were most severely impaired in stability and fibrillogenesis, alignment of the α2(I) chain appears to be critical.

### Temperature-Favored Self-Assembly of $Mn^{2+}$-DNA Aggregates

*Kornyshev, Leikin; in collaboration with Cherstvy*

Both collagen and $Mn^{2+}$-DNA self-assemble into well-organized fibers at elevated temperatures. Furthermore, the dependence of intermolecular forces in $Mn^{2+}$-DNA and collagen fibers on temperature and distance are qualitatively similar. Traditionally, such counterintuitive temperature dependence is attributed to the entropy gain associated with the release of structured water upon fiber assembly. However, our study of the physical mechanism of collagen fibrillogenesis suggested that the temperature dependence of the process is related to the entropy gain upon triple helix micro-unfolding in fibers. Based on these observations, we re-evaluated potential mechanisms of the temperature dependence in DNA aggregation, analyzing the possible contribution of counterion entropy. We described the interaction between DNA helices in terms of screened Coulomb forces and estimated the entropy gain upon DNA aggregation associated with $Mn^{2+}$ repartitioning between binding sites in minor and major grooves. The model rationalizes both the observed force-versus-separation and entropy-versus-separation curves, provided that the energy and entropy associated with transition of one $Mn^{2+}$ ion from minor to major groove are $\Delta E_{ads}$=3 to 5 $k_BT$, and $\Delta S_{ads}$=2 to 5 $k_B$, respectively. While these values appear to be reasonable, no experimental information that would allow their validation is currently available, and other interpretations of the observed force curves cannot be excluded at the present.

## SELECTED PUBLICATIONS

1. Bonnet-Gonnet C, Leikin S, Chi S, Rau DC, Parsegian VA. Measurement of forces between hydroxypropylcellulose polymers: temperature favored assembly and salt exclusion. J Phys Chem B. 2001;105:1877-1886.
2. Kornyshev A, Leikin S. Sequence recognition in the pairing of DNA duplexes. Phys Rev Lett. 2001;86:3666-3669.
3. Kornyshev AA, Leikin S, Malinin. Chiral electrostatic interaction and cholesteric liquid crystals of DNA. Eur Phys J E. 2002;7:83–93.
4. Kuznetsova N, McBride DJ, Leikin S. Osteogenesis imperfecta murine: interaction between type I collagen homotrimers. J Mol Biol. 2001;309:807-815.

5. Leikina E, Mertts MV, Kuznetsova N, Leikin S. Type I collagen is thermally unstable at body temperature. Proc Natl Acad Sci USA. 2002;99:1314-1318.

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